



**The Impact of Myeloid Derived Suppressor Cells on Vaccine
Immunogenicity in South African HIV-infected and Uninfected Mothers and
their Infants**

By

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DECLARATION

I **Elvis Banboye KIDZERU**, hereby declare that this thesis is based on my own original, unaided work (except where acknowledgements indicate otherwise). It is being submitted for the Degree of Doctor of Philosophy (PhD) in Clinical Science and Immunology at the Division of Immunology (DOI), Department of Pathology, in the Institute of Infectious Diseases and Molecular Medicine (IDM), the University of Cape Town, Cape Town, South Africa. Neither the whole work nor any part of it has been, is being, or is to be submitted for another degree or examination in this or at any other University. I authorize the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature:

Signed by candidate

Date: day of 15th March, 2016

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen presenting cells
BaP	Bordetella acellular Pertussis
BCG	Bacillus Calmette-Guérin
BF	Breast feeding
BP	<i>Bordetella Pertussis</i>
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CMI	Cell mediated immunity
CpG-ODN	Cytidine-phosphate-Guanosine oligodeoxynucleotide
CTL	Cytotoxic T lymphocytes
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DTaP	Diphtheria-Tetanus-acellular Pertussis vaccine (Paediatric formulation)
dTap	Diphtheria-Tetanus-acellular Pertussis vaccine (adult formulation)
DTP	Diphtheria-Tetanus-Pertussis vaccine
EBF	Exclusive breast feeding
ELISpot	Enzyme-Linked Immuno Spot
EPI	Expanded Programme on Immunization
FDA	Food and Drug Administration
GAVI	Global Alliance for Vaccine Initiative
GEE	Generalised estimation equation
HAART	Highly active antiretroviral therapy
Hep. B sAg	Hepatitis B surface antigen
HEU	HIV-exposed uninfected
HIV	Human immunodeficiency virus
HU	HIV-unexposed
ICS	Intracellular cytokine staining
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1 β	Interleukin-1beta
Ki67-PA	Ki67 proliferation assay
MDSC	Myeloid Derived Suppressor Cells
MF	Mix feeding

MHC	Major histocompatibility complex
NK	Natural killer
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PCV ₇	Pneumococcal Conjugate Vaccine
pDC	Plasmacytoid dendritic cells
PMA	Phorbol 12-myristate 13-acetate
PMTCT	Prevention of mother-to-child transmission
PRR	Pattern-recognition receptors
RPMI	Roswell Park Memorial Institute
RV	Rotavirus vaccine
Sags	Superantigens
SEB	Staphylococcal enterotoxin B
SIV	Simian immunodeficiency virus
SSA	Sub-Saharan Africa
TB	Tuberculosis
TcR	T cell receptors
Th	T helper
TLR	Toll-like receptor
TNF- α	Tumor necrotic factor-alpha
Treg	T regulatory
TT	Tetanus toxoid
WBA	Whole blood assay
WHO	World Health Organisation

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The Impact of Myeloid Derived Suppressor Cells on Vaccine Immunogenicity in South African HIV-infected and Uninfected Mothers and their Infants

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BACKGROUND

Each year over 4 million infants die from infections, of which many are vaccine-preventable. Young infants respond poorly to vaccines, but the basis of reduced immunity is controversial. We hypothesized that myeloid-derived suppressor cells (MDSC) that might be induced during gestation, would persist at birth leading to active suppression of infant-immune responses.

OBJECTIVE

We evaluated the ontogeny of MDSC and the effect of MDSC on vaccine immunogenicity during early life in South African infants and mothers, and in HIV-exposed uninfected (HEU) infants and HIV+ mothers.

METHODS

HIV-infected and uninfected mothers and their infants were recruited from Khayelitsha, Cape Town and followed-up for one year. In whole PBMC and after MDSC (CD15+) depleted, we measured BCG, Hepatitis B, Tetanus toxoid and *Bordetella pertussis* vaccine-specific CD4⁺ T cell proliferation by CFSE and IFN- γ responses using ELISpot assay.

RESULTS

MDSC frequency mothers and their infants were significantly higher at delivery, and decreased over time through 1 year of age. HIV+ mothers had a significantly higher MDSC frequency at one year postpartum, but there was no difference in MDSC frequency between their HIV-exposed and unexposed infants. In infants, MDSC depletion had variable effects on CD4⁺ T cell proliferation and IFN- γ production to deferent antigens. In both infants and mothers, MDSC depletion significantly increased responses to Tetanus and Hep B, but BCG responses were significantly lower after MDSC depletion in infants and mothers. HIV-exposure significantly reduced IFN- γ production to Tetanus toxoid vaccinations in infants.

CONCLUSION

High frequencies of MDSC are present at birth, but decrease with age in infants. Therefore MDSC effects on vaccine responses may be short-lived, and dependent on the type of antigen.

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1.1 Introduction

Immunization against vaccine-preventable infections is essential to reducing childhood morbidity and mortality. The complexity of the immune system of infants renders them susceptible to infectious diseases and makes induction of protective immunity via vaccines a challenge(Ota *et al.*, 2012). Neonates are thought to predominantly make type 2 cytokines, which include; interleukins (IL) IL-4, IL-5, and IL-13(Lambert *et al.*, 2005). This immunologic state may be beneficial *in utero*, but it is suggested to pre-dispose infants to severe infections. In contrast, Type 1 T Helper cells (Th1) that secrete interferons-gamma (IFN- γ), IL-2 and tumour necrosis factors (TNF), which are important for control of intracellular pathogens such as HIV, Tuberculosis, and Hepatitis B virus(Hassan, 2000), are impaired in neonatal responses to infections, and some vaccinations (Siegrist, 2001)(Makrigiannakis *et al.*, 2011). In addition altered T cell function in neonates, innate immune cells are different from those elicited later in life, and this may contribute to neonatal vulnerability to pathogens(Siegrist, 2001). It is not clear if the decreased ability to mount antigen-specific responses is due to an inherent immaturity of the effector cells and antigen-presenting cells (APC), or the functions of these cells are actively suppressed by foetal regulatory cells induced during gestation for tolerance(Gantt *et al.*, 2014)(Gervassi *et al.*, 2014). In order to contribute to developing more effective vaccination strategies that elicit better responses in infancy, it is helpful to understand neonatal immunological characteristics, including the impact of regulatory and suppressor cell populations. Recently in collaboration with our lab, a population of innate MDSC were discovered to be present in significantly high numbers in neonates, and could be responsible for some of the described infant T cell dysfunction(Gervassi *et al.*, 2014).

1.2 Myeloid-derived Suppressor Cells (MDSC)

Myeloid-derived suppressor cells are a heterogeneous population of immature, activated myeloid cells with immuno-suppressive function (Poschke *et al.*, 2012) (Gabrilovich *et al.*, 2012) (Ostrand-rosenberg *et al.*, 2012) (Pillay *et al.*, 2013). These cells, have been termed myeloid derived suppressor cells (MDSC) based on their myeloid origin and immunosuppressive nature (Gabrilovich *et al.*, 2007). MDSC can be best defined based on their immuno-suppressive effect on T cells (Greten *et al.*, 2011). MDSC are defined by phenotype into two subsets: granulocytic type (G-MDSC) that are often identified as CD11b⁺CD14⁻CD33⁺CD15⁺HLA-DR⁻, or the monocytic type (M-MDSC) described as CD11b⁺CD14⁺CD33⁺CD15⁻HLA-DR⁻ (Greten *et al.*, 2011). The definition of MDSC is challenging, and no consensus has been reached regarding the best markers in human studies (Damuzzo *et al.*, 2015). Immature myeloid cells generated in the bone marrow differentiate into mature, functional macrophages, dendritic cells, and granulocytes. In malignancy and pathologies involving persistent inflammation, there is accumulation and activation of immature granulocytic or monocytic myeloid cells expressing suppressive factors including, Arginase-1 (ARG), reactive oxygen species (ROS), and inducible nitric oxide synthase (NOS) (Gabrilovich *et al.*, 2012) (Ostrand-rosenberg *et al.*, 2012). ARG and NOS, are a substantial aspect of MDSC-mediated immune suppression. ARG and NOS share a common substrate L-arginine, which is metabolized to produce urea and L-ornithine (or NO and L-citrulline) respectively (Bogdan, 2001).

ARG depletes L-arginine which leads to extracellular or intracellular L-arginine starvation (Rodriguez *et al.*, 2007) (Rodriguez *et al.*, 2004). L-arginine starvation decreases CD3 zeta chain expression on T-cell receptors (TCR) and compromises TCR-mediated signalling (Zeng *et al.*, 2014) (Bronte *et al.*, 2005). NOS arrest T cell proliferation from G1 to G0 phase of the cell cycle inducing T cell apoptosis (Marigo *et al.*, 2008).

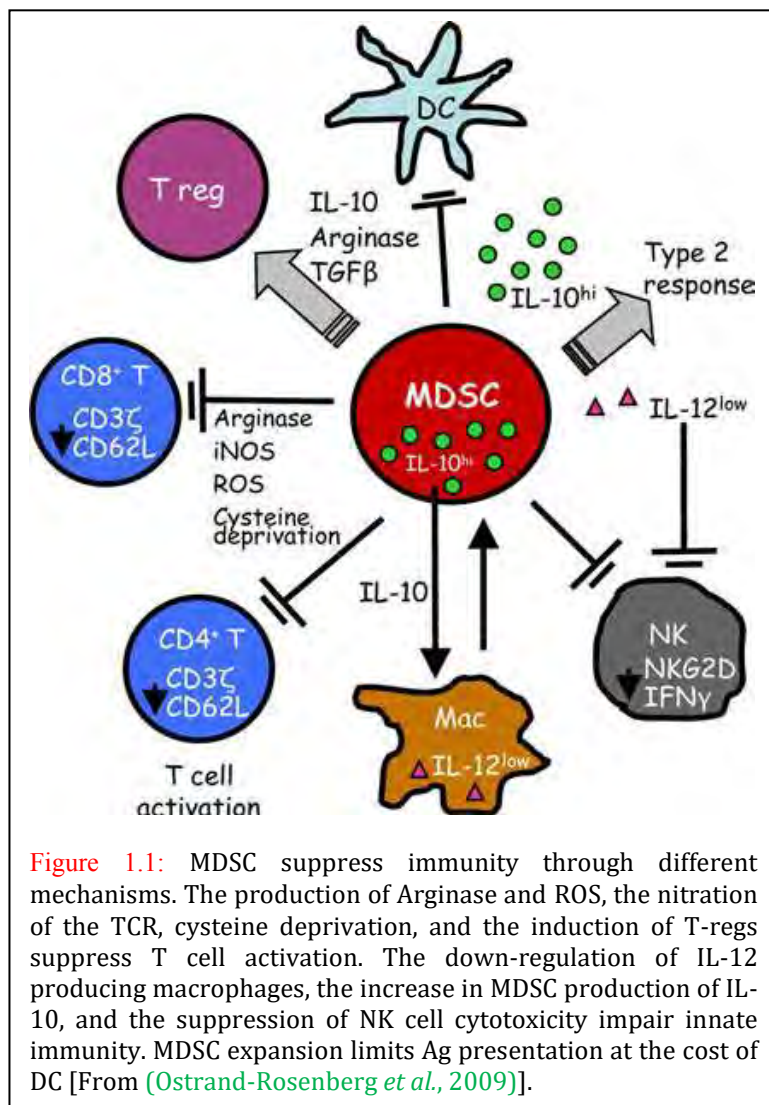
Immune suppression by MDSC is mediated through a variety of diverse mechanisms (Figure 1.1). In some cases, MDSC have been shown to inhibit T-cell responses in a NOS and ROS-dependent manner (Garg *et al.*, 2014).

In other cases, the suppressive mechanism by which MDSC mediate immune responses may be via induction of T-regs. In humans, MDSC have been reported to enhance differentiation of naïve $CD4^+$ T cells to T-regs (Hoechst *et al.*, 2011) (Brown *et al.*, 2008). Vollbrecht *et al.* and Garg *et al.* found that granulocytic and monocytic MDSC respectively induced the expansion of $CD4^+CD25^+FoxP3^+$ T-regs (Vollbrecht *et al.*, 2012) (Garg *et al.*, 2014).

The frequency of MDSC is low in healthy adults and 6-10 fold increased in cancer patients (Gabitass *et al.*, 2011).

While many studies on these cells has focussed on MDSC in murine cancer models and humans with malignancy (Poschke *et al.*, 2012), little is known about the role of MDSC in healthy people.

In healthy pregnancy, the immune environment in pregnant women during gestation is altered. Pregnant women are relatively immune compromised, which is thought to be due to the need for maternal-foetal tolerance (Nair *et al.*, 2015) (Serafini, 2013). High frequency of T regulatory cells



(T-regs) have been shown in pregnancy, and T-regs have been found to induce MDSC (Burt, 2013)(Gantt *et al.*, 2014)(Bansal, 2010)(Nair *et al.*, 2015)(Ostrand-rosenberg *et al.*, 2012). Indeed, pregnant women have recently been shown to have high MDSC frequency in peripheral blood (Rieber *et al.*, 2013). High frequency of T-regs as well as MDSC could be a physiologic phenomenon to maintain a healthy pregnancy via the induction of foeto-maternal tolerance, which if compromised could lead to miscarriage (Nair *et al.*, 2015)(Serafini, 2013). Nair *et al.* recently found that MDSC deficiency in the blood and endometrium of pregnant women was associated with early miscarriages (Nair *et al.*, 2015), and the important role MDSC play to maintain foeto-maternal tolerance may be detrimental postnatally as MDSC may persist after birth.

Since MDSC are found in high frequency in human neonates (Gervassi *et al.*, 2014)(Rieber *et al.*, 2013)(Jacobson *et al.*, 2011), it is plausible that this is due to delayed clearance of these cells postnatally, and that the persistence of these cells may contribute negatively in the ability of infants to respond to infections and vaccines.

1.3 MDSC and responses to infections and vaccination

Several factors are involved in the induction of MDSC including infections, inflammation, vaccines, adjuvants, and T-regulatory cells (Garg *et al.*, 2013)(Obermajer *et al.*, 2012)(Movahedi *et al.*, 2008)(du Plessis *et al.*, 2013)(Sui *et al.*, 2014)(Sinha *et al.*, 2012)(Guo *et al.*, 2012)(Vollbrecht *et al.*, 2012)(Verschoor *et al.*, 2013)(R. S. Tacke *et al.*, 2012)(Rieber *et al.*, 2013)(Cripps *et al.*, 2010)(Martino *et al.*, 2010). Increased frequency of MDSC have been associated with active tuberculosis disease, and after recent tuberculosis infection, and dampen T cell responses (du Plessis *et al.*, 2013)(Kumar *et al.*, 2014). IFN- γ secreting T cells have been shown to enhance MDSC levels (Guo *et al.*, 2012)(Cripps *et al.*, 2010)(Greifenberg *et al.*, 2009). Also, high IL-6 secretion in plasma enhances MDSC levels in HIV infected individuals (Garg *et al.*, 2013). MDSC have been linked to viral persistence and down regulation of CD8 zeta TCR expression in chronic Hepatitis C infection (Zeng *et al.*, 2014)(Cripps *et al.*, 2010). In a murine

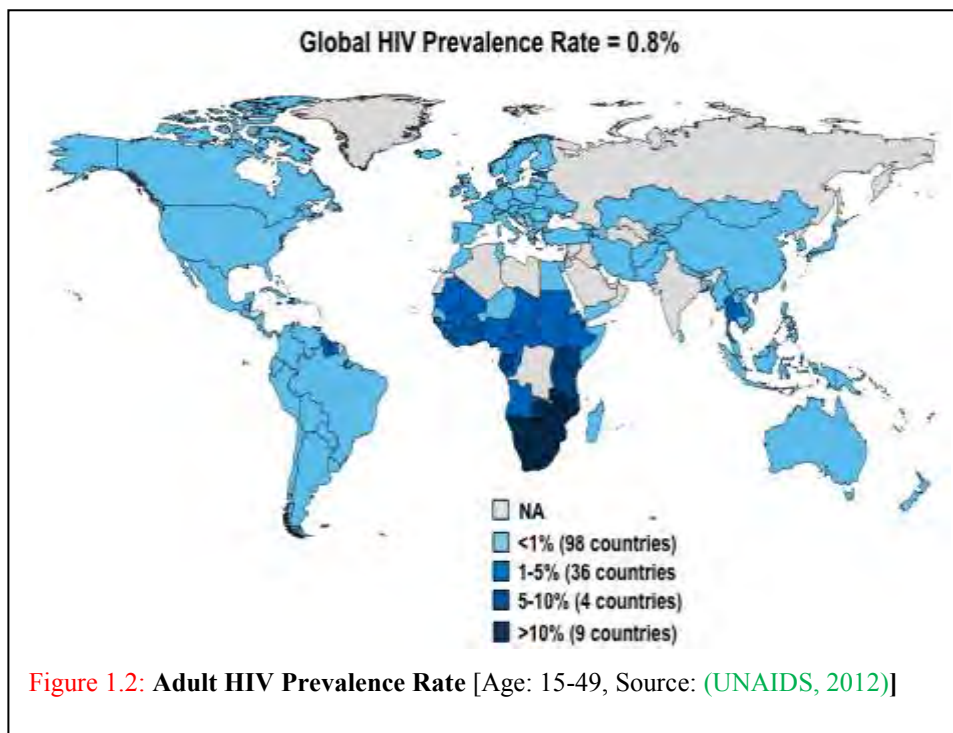
model, MDSC have been shown in Rotavirus-induced immunodeficiency and reduce lymphocyte responses leading to immune deficiency (Green *et al.*, 2013).

Although the suppressive effect of MDSC in several infection states have been well described (du Plessis *et al.*, 2013), (Tacke *et al.*, 2012), (Pallett *et al.*, 2015) (Weber, 2015) (Garg *et al.*, 2013) (Macatangay *et al.*, 2012), MDSC have also been shown to induce certain beneficial effects. Interferon gamma secreting MDSC have been shown to protect against invasive group A streptococcus infection (Matsumura *et al.*, 2012). Most recently, Pallett *et al.* showed that MDSC regulate hepatitis B liver immunopathology metabolically in hepatitis B infection (Pallett *et al.*, 2015) (Weber, 2015).

There is limited data on the effect of MDSC on immune responses to vaccines in infants. There is evidence of vaccine induced MDSC in adult macaques, which can dampen vaccine-induced immune responses (Sui *et al.*, 2014). Rieber *et al.* showed that MDSC cause alteration of both innate and adaptive immune responses in cord blood to non-specific stimulation (Rieber *et al.*, 2013). We have also shown that in neonates, MDSC dampens T cell responses in cord blood to polyclonal stimulation (Gervassi *et al.*, 2014). There is little or no information about the effect of MDSC on immune responses to specific infections and vaccinations in infants.

1.4 Infants and maternal Human Immuno-deficiency Virus (HIV)

Human Immuno-deficiency virus (HIV)-1 infection is still a major pandemic. It causes Acquired Immunodeficiency Syndrome (AIDS) and is one of the world's most serious health and development challenge(UNAIDS, 2012)(UNAIDS, 2011b). Currently, approximately 34 million people have died of AIDS-related causes since the first cases were reported in 1981 (The Henry J. Kaiser Family Foundation/UNAIDS, 2012)(UNAIDS, 2011a). Almost all cases of HIV (97%) reside in low- and middle-income countries, particularly in



SSA(The Henry J. Kaiser Family Foundation/UNAIDS, 2012). Over 3.5 million women of childbearing age are infected annually with HIV in SSA and large numbers of children are born to HIV-positive women(UNAIDS, 2012). Figure 1.2 depicts global HIV prevalence rates.

It is estimated that there are more than 2 million children infected with HIV-1 worldwide and that more than 1800 new HIV infections occur daily from mothers to infants(UNAIDS/WHO, 2006). Transmission rate can be reduced to as low as 1-2% with the use of antiretroviral therapy (ART) (UNAIDS, 2012). Through Prevention of Mother to Child Transmission (PMTCT) programs, this leaves an ever-increasing number of HIV-exposed, uninfected (HEU) infants.

1.4.1 MDSC and Human Immuno-deficiency Virus (HIV)

The immune system of HIV infected is compromised and makes them prone to common infections and altered responses to vaccination(Epalza *et al.*, 2010). High frequencies of monocyte derived MDSC levels have been shown in the peripheral blood mononuclear cells (PBMC) of HIV positive individuals compared to healthy controls(Qin *et al.*, 2013), and there is evidence that MDSC suppress T cell function in HIV-1 infection. Based on the elevated levels of MDSC in HIV infection, this suggests that the surge in MDSC levels may be a mechanism by which HIV mediates suppression of the immune system.

HIV infection has long been causally related to T cell activation, considered to be the central mediator of HIV pathogenesis(Hunt, 2007)(Andrieu *et al.*, 2014). High T cell activation levels have been shown to predict more rapid disease progression in untreated patients and decreased treatment-mediated CD4⁺ T cell gains during antiretroviral therapy. This may be the primary aspect distinguishing pathogenic from non-pathogenic HIV infection(Vollbrecht *et al.*, 2012)(Lieberman *et al.*, 2010)(O'Connell *et al.*, 2011). It is therefore possible that MDSC are induced in response to the extreme immune activation during HIV infection(Qin *et al.*, 2013)(Gama *et al.*, 2012)(Nagaraj *et al.*, 2013). On the other hand, MDSC may suppress antiviral responses and therefore play a role in AIDS pathogenesis(Gama *et al.*, 2012)(Garg *et al.*, 2013). HIV-1 gp120 has been shown to induce expansion of MDSC *in vitro*? and hence suppress immunity to anti-CD3CD28, and these data suggest a phenotype that may be linked to viral replication(Garg *et al.*, 2013). Further, it has been shown that HIV-1 gp120-induced MDSC dampens CD4⁺ and CD8⁺ T cell responses in a cell-to-cell contact fashion(Garg *et al.*, 2013). Chronic progressive HIV-1 infection has been shown to be associated with increased levels of MDSC(Vollbrecht *et al.*, 2012). The HIV-1 gp120-induced MDSC have been shown to enhance T-regs expansion and IL-10 production by CD4⁺ T cells(Garg *et al.*, 2013).

In a non-human primate macaque model, vaccine-induced MDSC have been shown to dampen protective immunity to SIV in vaccinated macaques(Sui *et al.*, 2014). However, little is known about the effect of MDSC in HIV-exposed uninfected infants.

1.5 HIV-exposure in infants

The absence of an effective HIV vaccine requires innovative approaches, immunology of several immune cells to provide new information into vaccine development. While the immunobiology of regulatory T cells has been studied intensively in non-human primates(Connor *et al.*, 2007)(Bumseok *et al.*, 2009)(Gama *et al.*, 2011) and HIV-unexposed (HEU) infants(Mold *et al.*, 2009), there is little data about how regulatory T cells and MDSC could hinder vaccine responses in HEU especially in sub-Saharan Africa (SSA)(Legrand *et al.*, 2006).

HIV-exposed but uninfected (HEU) infants are infants born to HIV-infected mothers but escape HIV infection(Afran *et al.*, 2013). An estimated two million infants are exposed *in utero* to HIV of which an approximately 1.5 million are HIV uninfected. Moreover, due to the high maternal HIV prevalence in SSA coupled with the successful and ever improving prevention of mother-to-child transmission (PMTCT) programs in many parts of the world(Mbori-ngacha *et al.*, 2001), there is a growing proportion of HEU infants (Filteau, 2009).

1.5.1 Alterations in the immune system of HEU infants

HIV-exposed but uninfected infants are a group of vulnerable infants with increase morbidity and mortality compared to the HIV-unexposed (HU) infants(Epalza *et al.*, 2011)(Kuhn *et al.*, 2006)(Mussi-pinhata, 2006)(Marinda *et al.*, 2007)(Kidzeru *et al.*, 2014). Maternal immunosuppression due HIV infection tends to have adverse influence on the health of infants in addition to the risks of vertical HIV transmission(Kuhn *et al.*, 2006). However, Zambian HIV exposed infants who remained uninfected after perinatal or early breastfeeding-related HIV exposure were of high risk of mortality and morbidity (pneumonia and/or sepsis) during the first few months of life regardless of their maternal disease stage or Socioeconomic status(Kuhn *et al.*, 2006).

Skin/mucous membrane infections and respiratory tract infections are common infections observed in HEU infants more often than HIV-unexposed infants(Mussi-pinhata, 2006)(Kidzeru *et al.*, 2014). Also, the incidence of Group B Streptococcal infection is significantly higher in

HEU than in the unexposed infants (Epalza, 2010). There are data documenting different responses to vaccine immunogens (van Rie *et al.*, 2006) (Jones, 2011) (Mazzola, Marcos, & Abramczuk, 2011), and an increased risk of *Pneumocystis jirovecii* pneumonia an opportunistic infection (Marinda *et al.*, 2007) in HEU infants. HIV-exposure has been shown to impair cellular immune responses in infants (Rie *et al.*, 2006) (Mazzola *et al.*, 2011), this impairment has also been shown in early life to humoral immune responses to vaccine but developed with age (Jones *et al.*, 2011).

HEU infants have increased immune activation and we have also shown increased T cell proliferation to antigens (Kidzeru *et al.*, 2014), possibly as a result of *in utero* exposure to HIV antigens and/or antigens related to other infections in HIV-infected mothers (Rich *et al.*, 1997). Also, elevated levels of pro-inflammatory cytokines are present in HEU infants (Hygino *et al.*, 2008). Both immune activation and a proinflammatory cytokine profile are known to induce MDSC (Davenport *et al.*, 2007) (Lieberman *et al.*, 2010) (Hunt, 2007). However, the role that MDSC play in the immunological abnormalities caused by HIV exposure in infants has not been investigated.

To improve the morbidity of this immunologically vulnerable group of infants, we need to understand the mechanisms of their altered immune responsiveness to determine optimal vaccination and disease prevention strategies.

1.5.2 Cellular immunity in HEU infants

We showed that HIV-exposure is associated with increased T cell proliferation to BCG, but that these proliferating cells are poor cytokine producers (Kidzeru *et al.*, 2014). In a study by Mazzola *et al.*, it was found that compared to HIV-unexposed infants, very young HEU infants produced less IFN- γ and TNF- α in response to BCG, but the level of IFN- γ produced in response to BCG increased with age suggesting there may be defects in immune responses early in life in HEU infants (Mazzola *et al.*, 2011). We have also shown that in HEU infants, delaying BCG vaccination until 8 weeks of age enhances BCG-specific T cell responses (Tchakoute *et al.*, 2014).

1.6 Vaccination and immunity

Before the Expanded Programme on Immunization (EPI) was launched in 1974, fewer than 5% of the world's children were immunized during their first year of life against the six killer

Table 1.1: The EPI vaccination schedule used in South Africa;(1) BCG given at birth, (2) DTaP-IPV/Hib primed at 6 weeks, first, second boosters at 10, 14 weeks and third booster at 18 months of life and further at 6 and 12 years of age (Source: <http://www.amayeza-info.co.za/>)

Vaccine Schedules for South Africa for 2015 Compiled by Amayeza Info Services' Vaccine Helpline:					
Age of child	EPI schedule (6-10-14 wks)	Private (6-10-14 wks)	Private (6-10-14 wks + HepB at Birth)	Age of child	Option 3 in Private (2-4-6 months)
At birth	OPV(0)	OPV(0)	OPV (0)	At birth	OPV(0)
	BCG	BCG	BCG		BCG
			HBV ¹		
6 weeks	OPV(1)	OPV(1)	OPV(1)	2 months	OPV(1)
	RV (1)	RV (1)	RV (1)		RV (1)
	PCV(1)	PCV(1)	PCV (1)		PCV (1)
	DTaP-IPV/Hib (1) ⁴ + HBV(1), or DTaP-IPV-Hib-HBV (1) ⁴	DTaP-IPV-Hib-HBV (1) ²	DTaP-IPV-Hib-HBV (1) ² or DTaP-IPV-HBV/Hib (1) ²		DTaP-IPV-Hib-HBV (1) or DTaP-IPV-HBV/Hib (1)
10 weeks		RV ² (2)	RV ² (2)	3 or 4 months	RV ² (2)
		PCV (2)	PCV (2)		PCV (2)
	DTaP-IPV/Hib (2) + HBV(2) or DTaP-IPV-Hib-HBV (2)	DTaP-IPV/Hib (2) + HBV (2) or DTaP-IPV-Hib-HBV (2)	DTaP-IPV-Hib-HBV (2) or DTaP-IPV-HBV/Hib (2)		DTaP-IPV-Hib-HBV(2) or DTaP-IPV-HBV/Hib (2)
14 weeks	RV (2)	RV ² (2 or 3)	RV ² (2 or 3)	4 or 6 months	RV ² (2 or 3)
	PCV(2)	PCV (3)	PCV (3)		PCV (3)
	DTaP-IPV/Hib (3) + HBV(3) or DTaP-IPV-Hib-HBV (3)	DTaP-IPV/Hib (3) + HBV (3) or DTaP-IPV-Hib-HBV (3)	DTaP-IPV-Hib-HBV (3) or DTaP-IPV-HBV/Hib (3)		DTaP-IPV-Hib-HBV(3) or DTaP-IPV-HBV/Hib (3)
9 months	Measles (1)	Measles (1)	Measles (1)	9 months	Measles (1)
	PCV(3)	MCV (1)	MCV (1)		MCV (1)
12-15 months		PCV (4) ⁴	PCV (4) ⁴	12-15 months	PCV (4) ⁴
		MMR (1)	MMR (1)		MMR (1)
		Varicella ³ (1)	Varicella ³ (1)		Varicella ³ (1)
		Hepatitis A (repeat 6 months later)	Hepatitis A (repeat 6 months later)		Hepatitis A (repeat 6 months later)
18 months		MCV (2)	MCV (2)	18 months	MCV (2)
	DTaP-IPV/Hib (4) or DTaP-IPV-Hib-HBV (4)	DTaP-IPV/Hib (4) or DTaP-IPV-Hib-HBV (4) or DTaP-IPV-HBV/Hib (4)	DTaP-IPV/Hib or DTaP-IPV-Hib-HBV (4) or DTaP-IPV-HBV/Hib (4)		DTaP-IPV/Hib or DTaP-IPV-Hib-HBV (3) or DTaP-IPV-HBV/Hib (3)
	Measles (2)				
5-6 years	Td vaccine (6 years)	DTaP or Tdap-IPV	DTaP or Tdap-IPV	5-6 years	DTaP or Tdap-IPV
		MMR (2)	MMR (2)		MMR (2)
		Varicella (2)	Varicella (2)		Varicella (2)
9 years	HPV ⁶	HPV ⁷ (from 9 years)	HPV ²⁸ (from 9 years)	9 years	HPV ⁷ (from 9 years)
12 years	Td vaccine	Tdap-IPV ⁸	Tdap-IPV ⁸	12 years	Tdap-IPV ⁸

References for vaccine schedule:

- Expanded programme of immunisation (EPI) (From April 2009)
- Package inserts: Infanrix-Hexa, Hexaxim, Prevenar, Varilrix, Gardasil, Cervarix, Adacel quadra, Synflorix, Boostrix Tetra, Priorix Tetra, Trimovax, Priorix, Pentaxim, Rotatag, Rotarix, Menactra.
- Personal communication with GSK (Upper age limit of Infanrix-Hexa®)
- CDC pink book <http://www.cdc.gov/vaccines/Pubs/pinkbook/pink-chapters.htm>

diseases in that same year; polio, diphtheria, tuberculosis, pertussis (whooping cough), measles and tetanus(UNICEF/WHO, 2011). Today, immunization is been carried out effectively throughout the world, with some vaccines have been prioritized in areas where the disease burden is of major public health concern(CDC, 2005). Table 1.1 shows the EPI vaccination schedule used in South Africa.

1.6.1 Vaccination

1.6.1.1 Bacillus Calmette-Guérin (BCG)

BCG, a live attenuated *Mycobacterium bovis* vaccine, is almost universally given in SSA, where the brunt of the global paediatric HIV burden is concentrated. BCG is usually given at birth; its coverage worldwide is estimated at over 100 million doses per year, resulting in vaccination of 75% of infants born in 2002 globally. In HIV-uninfected children, BCG is safe, efficacious and cost-effective against disseminated tuberculosis(Trunz *et al.*, 2006). BCG can elicit potent type 1 responses and effective cytotoxic T cell (CTL) responses in infants, even when given at birth.

We do not know, however, how efficacious BCG is in HEU infants, although some evidence suggests that it may not be as immunogenic as in HU infants(Rie *et al.*, 2006)(Mazzola *et al.*, 2011). Recently in our lab we found that BCG induces significantly higher T-cell proliferation in HEU than in HU infants(Kidzeru *et al.*, 2014). Also, we showed that in HEU infants, delaying BCG vaccination until 8 weeks of age enhance robust BCG-specific T cell responses(Tchakoute *et al.*, 2014). Owing to the fact that neonates are potent type 2 cytokines (IL-4, IL-5, IL-13) producers(Lambert *et al.*, 2005) important for B cell development, neonatal T cell response to HIV and BCG vaccination may be an ideal model for an HIV vaccine study. BCG is widely known to elicit Th1 responses and although the correlate of protection is unknown, induction of IFN- γ is thought to be important in protection against TB(Beveridge *et al.*, 2008)(Mansoor *et al.*, 2010)(WHO, 2009)(Hanekom *et al.*, 2008).

1.6.1.2 Tetanus toxoid (TT)

Tetanus is a deadly infectious disease for which immunization is available in EPI schedules of most countries worldwide and tetanus boosters are recommended every 5 – 10 years for everyone (Aziz, 2010). Tetanus results from infection with *Clostridium tetani*, a commensal bacterium in the gut of humans and domestic animals which is found also in soil. It is not the presence of the bacteria which causes disease, but the toxins that are produced by the bacteria under anaerobic conditions (CDC, 2005). These toxins can be spread through the blood vessels and finally affect the nervous system causing tetanic muscle contraction and pain. The condition is extremely painful and potentially lethal (Aventis Pasteur, 1989) (Gaublomme, 2007).

In South Africa, immunization against tetanus is carried out by the administration of DTaP in infants and dTap in adults, both containing Tetanus toxoid with aluminium hydroxide as an adjuvant, and the vaccine is available in combination with other antigens as well as alone. Tetanus vaccine is available on the EPI schedule in many countries for children at 6, 10 and 14 weeks and at 18 months of age (CDC, 2012) (Gaublomme, 2007). Tetanus toxoid vaccination is widely known to elicit antibody responses and the correlate of protection is known to be the induction of serum IgG (Claire-anne Siegrist, 2008), and this has been shown in HIV-exposed uninfected infants (Jones *et al.*, 2011). Therefore, little is known about tetanus toxoid induced T cell responses. However, in healthy children tetanus toxoid vaccine responses have been shown to be initially Th2 polarized (Rowe *et al.*, 2000), also, Cooper *et al.* have shown impaired Tetanus-specific cellular immune responses after tetanus vaccination (Cooper *et al.*, 1998). Cellular immune responses to tetanus vaccination have been shown to contribute to long-term immunity (Graziani *et al.*, 2013).

1.6.1.3 Acellular Bordetella Pertussis (aP)

Pertussis, or whooping cough, is an acute infectious disease caused by the bacterium *Bordetella pertussis*. Pertussis was one of the most common childhood diseases in the 20th century and a major cause of childhood mortality in the United States. Before the availability of pertussis vaccine in the 1940s, more than 200,000 cases of pertussis were reported annually. Since the

widespread use of pertussis vaccination began, incidence has decreased more than 80%(Mooi et al., 2009)(States, 2008). Two forms of vaccines have been used (i) Whole-cell pertussis vaccine (wP) and (ii) acellular Pertussis vaccine (aP). The wP vaccine is composed of a suspension of formalin-inactivated *Bordetella pertussis* cells. Whole-cell Pertussis vaccine is 70%-90% effective in preventing serious pertussis disease. However, its protection decreased with time, resulting in little or no protection 5 to 10 years following the last dose. Reactions such as redness, swelling, and pain at the injection site, fever and other systemic events were relatively common with wP (CDC, 2005)(Demirjian et al., 2009). This raised concerns about its safety and led to the development of more purified (acellular) Pertussis vaccines that was associated with a lower frequency of adverse reactions. South Africa is the only African country using aP, the rest are using wP.

Acellular Pertussis vaccines are subunit vaccines that contain purified, inactivated components of *Bordetella pertussis* cells(Sato, 1999). Several acellular Pertussis vaccines have been developed for different age groups; these contain different Pertussis components in varying concentrations which include; the paediatric formulation (DTaP) often available in combination with other antigens [Infanrix hexa™ (DTaP-hepB-IPV-Hib); Infanrix-IPV™ (DTaP-IPV); Infanrix Penta™ (DTaP-hepB-IPV)]. The adolescent and adult formulation (dTAp) has four types available containing reduced Pertussis antigen content in comparison to the vaccines for young children [Boostrix™ (dTAp) and Boostrix-IPV™ (dTAp -IPV), and Adacel™ (dTAp) and Adacel Polio™ (dTAp-IPV) (available since 2005)](NCIRS, 2009). Pertussis vaccine is available on the EPI schedule in many countries for children at 6, 10 and 14 weeks and at 18 months of age(Gebhart, 1980). An adolescent booster dose is available via school-based programs at 12-17 years of age which vary by countries(CDPH, 2010)(NCIRS, 2009). There is some data about *Bordetella pertussis* induced T cell responses, Ross et al. have shown that Th1 and Th17 both contribute to protective immunity by wP immunization but aP induce Th2 and Th17 cells but weak Th1 responses(Ross et al., 2013). Also, long term pertussis-specific T cell responses were observed after primary vaccination in HIV-unexposed infants(Esposito et al., 2001). We recently showed that pertussis vaccination caused equal T cell proliferation in both HIV-unexposed and HIV-exposed uninfected infants(Kidzeru et al., 2014). *Bordetella pertussis* vaccination is known to

elicit antibody and CD4⁺ T cell responses, and the correlate of protection is known to be the induction of serum IgG (Claire-anne Siegrist, 2008).

1.6.1.4 Hepatitis B (Hep B)

Hepatitis B virus (HBV) is the major cause of cirrhosis and liver cancer resulting in approximately 620,000 deaths annually worldwide (Goldstein *et al.*, 2005). Infections acquired during the perinatal period, in early childhood (<5 years old), and ≥5 years of age accounted for 21, 48, and 31% of Hepatitis B deaths, respectively (Goldstein *et al.*, 2005). Two major modes of HBV transmission occur during infancy;

- (1.) Perinatally from an infected mother during delivery, and
- (2.) From infected household contact to the infant.

Perinatal transmission accounts for approximately 21% of HBV-related deaths globally (Goldstein *et al.*, 2005). Transmission of HBV is prevented through immunization (Lu *et al.*, 2008); universal neonatal and infant hepatitis B vaccine, a sub-unit/conjugate vaccine which induces antibody against hepatitis B surface antigen (anti-HBs), has proved to be highly effective against HBV infection (YEN *et al.*, 2007). In 1992, the World Health Organization (WHO) recommended all countries to introduce hepatitis B (Hep B) vaccine into national EPI schedule by 1997 (CDC, 2007), this resulted in a significant decrease in prevalence of chronic HBV infection in most parts of the world (EI-HWEI *et al.*, 1997) (Chen *et al.*, 1999) (Su *et al.*, 2007). It is estimated that without vaccination, 64.8 million would become HBV-infected and 1.4 million would die from HBV-related disease since 2000. In countries with high percentage of HBV infection, WHO recommends administration of the first dose of Hep B vaccine <24 hours after birth to prevent perinatal HBV transmission (CDC, 2008) and also provides early pre-exposure protection to infants born to HBV uninfected mothers if exposure were to occur and the risk of developing chronic HBV infection is greatest and consequently death (Mast *et al.*, 2005).

However, administration of Hep B vaccine at birth is not feasible in developing countries where Hepatitis B is highly endemic, as is a practice that often results in missed post-exposure prophylaxis of infants (Mast *et al.*, 2005). In South Africa, Hep B vaccine is first administered at 6 weeks of age when EPI programs in most developing countries initiate administration of other vaccines to infants (Department of Health, 2009). Hep B vaccination is widely known to elicit antibody responses and the correlate of protection is known to be the induction of serum IgG (Claire-anne Siegrist, 2008). The current Hep B vaccine has been shown to be highly immunogenic in infants (Wang *et al.*, 2011). Few studies have looked at Hepatitis B humoral immune responses in HIV-exposed uninfected infants, (Rustein *et al.*, 1994) and little is known about cellular immunity to Hepatitis B in this vulnerable group. Mizukoshi *et al.* showed that Hep B vaccine immune responses were more stronger during acute, self-limiting and after recovery than during chronic hepatitis B infection (Mizukoshi *et al.*, 2004). Hep B vaccination known to elicit antibody responses (Claire-anne Siegrist, 2008), and this has been shown in HIV-exposed uninfected infants (Jones *et al.*, 2011) (Abramczuk *et al.*, 2011). It should be noted that HIV-infected mothers are more likely to transmit HBV to their neonates than HIV-negative women.

Hence, HIV exposure together with high frequency of MDSC in neonates could hinder vaccine immune responses in South African infants. In this study we will look at the quantity and ontogeny of MDSC in infants and how MDSC influence cellular immune responses to vaccines critical for providing information towards protection of infants from infectious diseases. *The ultimate goal is to design interventions to improve the health of infants, especially HEU infants, and to inform the design of a neonatal HIV vaccine.*

1.7 Introduction to the thesis

Hypothesis

We hypothesise that MDSC, present during gestation for fetomaternal tolerance, might persist into infancy, leading to active suppression of infant immune responses, which wanes over time. We further hypothesize that due to heightened MDSC in HIV-infection, HEU infants may have higher frequencies of MDSC, and that MDSC may be one contributing factor to their altered immunity.

Aims

We aim to evaluate the effect of MDSC on immune responses to EPI vaccines. To this end we have three specific objectives:

1. To evaluate the quantity and ontogeny of MDSC in infants and their mothers from birth to one year postpartum

Hypothesis

The prevalence of MDSC will diminish with age and become comparable to that of adults by one year of life. MDSC frequency will be high immediately after delivery in mothers, and decrease by one year postpartum. MDSC frequency will be lower in HU infants and HIV-negative mothers, compared to the HEU and HIV-positive infants and mothers respectively.

2. To assess the effect of MDSC on T cell proliferative responses to vaccines in infants and their mothers from birth to one year postpartum

Hypothesis

MDSC inhibit proliferative responses to vaccination in infants and their mothers. T cell proliferation will be lower in infants exposed to HIV and mothers infected with HIV due to the effects of higher frequencies of MDSC.

3. To assess the effect of MDSC on cytokine production to vaccines in infants and their mothers from birth through one year postpartum

Hypothesis

MDSC will inhibit cytokine in response to vaccination in infants and their mothers. Cytokine responses will be lower in infants exposed to HIV and mothers infected with HIV due to the effects of higher frequencies of MDSC.

Finally, to all the objectives, we aim to evaluate the effect of HIV-exposure or infection on MDSC frequency and function i.e. on all the above parameters.

Rationale

Studies focus on MDSC in murine cancer models and humans with malignancy. High frequencies of MDSC are found in peripheral blood of healthy neonates. Yet little is known about the effect of these MDSC on vaccine immune responses in infants. HIV-exposed infants display relative immune deficiencies. Understanding the impact of MDSC on immune responses of HIV-exposed infants will contribute to guide with design of neonatal HIV vaccines and other vaccines for this vulnerable group of infants.

1.8 References

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CHAPTER 2

COHORT CHARACTERISTICS

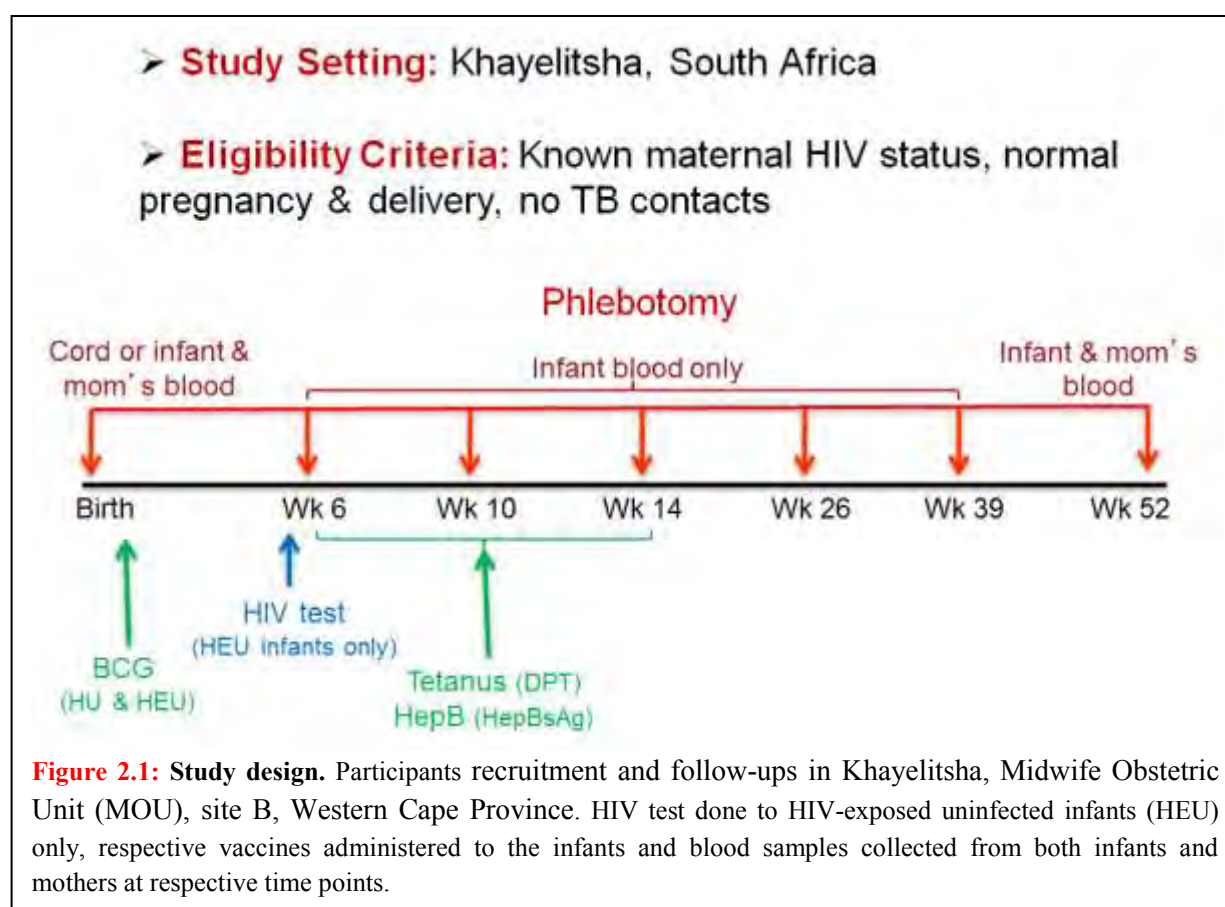
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2.1 Study design

This was a prospective longitudinal observational cohort study.

The outcome measures were MDSC quantity and ontogeny, as well as T cell proliferation and IFN- γ production in response to Bacillus Calmette-Guérin (BCG), tetanus toxoid (TT), Bordetella pertussis (BP) and Hepatitis B surface antigen (Hep. B) with and without MDSC in infants at, 6, 10 and 14 weeks; and at 6 and 9 months and 1 year of age (Figure 2.1).

This study was exploratory, since the true variance of MDSC frequency during infancy was



unknown. As our primary outcome, using estimates based on our preliminary cord blood and cross-sectional data during infancy, with a sample size of 100 infants, the study had an 80% power (2-sided, $\alpha = 0.05$) to detect an inverse correlation of at least 0.3 between MDSC frequencies at the time a given vaccine was administered and immune responses to that vaccine, such as antigen-specific T cell responses. All of these estimates were highly conservative,

however, as they assumed comparisons at only a single time point. Additional power was obtained using longitudinal data from all infants.

2.1.1 Participants and blood collection

This thesis is censored after 70 infants-mother pairs were enrolled into the cohort. Participants were mothers and their infants, regardless of HIV infection status. After informed consent was obtained, whole blood and/or cord blood of neonates, as well as whole blood from mothers was collected. Infants were followed up at 6, 10 and 14 weeks; and at 6 and 9 months; and finally at one year, and blood was obtained at these visits prior to vaccinations where applicable. In addition, maternal blood was collected at one year postpartum. The participant recruitment process involved completion of a screening form for eligibility assessment (see section 2.1.3) after informed consent was obtained. Physical examination including knowledge about maternal HIV status and health, infant gestational age, maternal history on antenatal clinic visits during gestation, parameters such as gestational diabetes, eclampsia, preeclampsia and placental abruption. The participant contact form was completed for follow up reminders.

2.1.2 Recruitment of participants

2.1.2.1 Study setting

Infants-mother pairs were recruited from Khayelitsha at the Midwife Obstetric Unit (MOU) at site B, Western Cape Province. Khayelitsha is historically an informal settlement located 56 km from the centre of Cape Town, with an estimated population of 500 000 (Nelson, 2014) (van Pletzen *et al.*, 2013). Antenatal (ANC) HIV seroprevalence rose from 19.3% in 2000 to 29% in 2009, and to 37% in 2011 and is the highest in the Western Cape (Stinson *et al.*, 2014) (Nelson, 2014). Site B has good existing research capacity and with established prevention of mother-to-child transmission (pMTCT) and pediatric antiretroviral (ARV) clinics (Stinson *et al.*, 2014). The mother to child transmission (MTCT) rate in the district as a whole was 2.7% in 2011 (Goga *et al.*, 2014). The Western Cape has one of the highest tuberculosis (TB) rates globally, with an infant TB rate estimated (culture-confirmed) at 1596 cases per 100,000 population among HIV-infected infants (95% CI, 1151-2132) and 66 cases per 100,000 population among HIV-uninfected infants (95% CI, 56-75) (Hesseling *et al.*, 2009). BCG and oral polio vaccine (OPV)

are routinely given at birth. Since April 2009, infants receive Rotavirus vaccine (RV) at 6 and 14 weeks and three doses of Diphtheria-Tetanus-aPertussis(DPT)-iPV/Hib, Hepatitis B, and pneumococcal conjugate (PCV) vaccinations at 6, 10 and 14 weeks of age (Department of Health, 2009) (Table 1.1)(Figure 2.1). In South Africa, pregnant women are not routinely given Tetanus toxoid vaccine boosters during gestation.

2.1.3 Exclusion criteria and HIV testing

2.1.3.1 Exclusion criteria

For both groups of infants, exclusion criteria included the following;

1. Mother was not willing and able to give consent
2. Baby weighed less than 2.4 kg
3. Baby was not healthy (presented with sepsis/ convulsions/ asphyxia/ severe respiratory distress/ severe congenital abnormality)
4. Mother or anyone in her house had known TB or a cough; then she was referred to the TB clinic.
5. Mother was planning to move herself or the infant away from Khayelitsha in the next 12 months
6. Prematurity (i.e. <36 weeks)
7. Mother was younger than 18 years

Additionally for the HIV-exposed infants, eligibility was based on the following;

1. Infant HIV DNA PCR test was negative at 6 weeks after birth

2.1.3.2 HIV testing

- a. For HIV-exposed infants, HIV testing was performed routinely by the pMTCT program at 6 weeks of age and, if breastfeeding, at 6 weeks after cessation of breastfeeding or at 9 months, whichever was earliest. Infants were excluded if they test positive for HIV infection.
- b. If the mother's HIV status was positive, the infant was tested for HIV infection according to local standard of care and if positive, was excluded from further follow-up and from the analysis. Any infected infants were immediately referred for HIV treatment and care.

2.2 Characteristics of the cohort

We evaluated the characteristics of 70 mother-infant pairs recruited, which included 24 HIV-infected women and their exposed uninfected (HEU) infants, and the remainder (46) HIV-negative women and their unexposed (HU) infants. In total 70 mother-infant pairs were recruited at delivery, 39 followed up at 6 weeks, 32 at 10 weeks, 30 at 14 weeks, 20 at 6 months, 15 at 9 months and lastly 14 infant-mother pairs completed the study at one year postpartum.

Among the infants there were 30 female (12 HEU and 18 HU) and 40 male [12 HEU and 28 HU], there was no significant difference between HIV-exposure groups ($p=0.383$) (Table 2.1).

Among all the parameters recorded, birth weight ($p=0.019$), weight at 6 months ($p=0.048$), and the weight at 9 months ($p=0.007$), were all significantly lower in the HEU infants. Also, a significantly lower frequency of HEU infants were breastfed (BF) at birth ($p=0.004$), 6 weeks ($p=0.022$), 14 weeks ($p=0.031$) and at 6 months ($p=0.007$) compared to HU infants (Table 2.1). The percentage (%) of HEU versus HU infants that were breastfed (BF) at 10 weeks [i.e. 66.7% versus 86.2%, $p=0.099$], at 9 months and at one year were not significantly different (Table 2.1).

The median and the interquartile range (IQR) of the weight of the HEU versus HU infants at 6 weeks [i.e. 4.3 (3.7-4.5) Kg versus 4.2 (4.2-4.8) Kg, $p=0.258$] as well as the weight at 10 weeks, 14 weeks and one year were not significantly different (Table 2.1). Also, there were no significant differences in the median gestational age between groups, as well as the percentage of common infections between HEU compared to the HU infants at all-time points by history (Table 2.1).

All mother infected with HIV were on antiretroviral (ARV) therapy, as well as in the prevention of mother to child transmission (PMTCT) program during pregnancy. Also, all infants born to HIV infected mothers were given nevirapine for at least 6 weeks.

Table 2.1: Characteristics of the cohort between HEU versus HU infants at 6 and 14 weeks of life;
the read outs for each parameter is indicated as medians and interquartile ranges (IQR) as well as in percentages (%) and p-values with red fonts illustrate parameters that were significantly different between HEU vs. HU infant groups i.e. p-values<0.05, statistical significance was obtained using **Rank sum, *Chi square or ^{\$}Fisher's exact tests.

Parameters	HIV-Unexposed (HU) Infants n=46	HIV-exposed Uninfected (HEU) Infants n=24	p-value
Gender Male/Female (%)	28/18 (70%/60%)	12/12 (30%/40%)	0.383*
Median birth weight (Kg) (IQR)	3.2 (3.0-3.5)	3.0 (2.8-3.1)	0.019**
Median weight at 6 weeks (Kg) (IQR)	4.2 (4.2-4.8)	4.3 (3.7-4.5)	0.258**
Median weight at 10 weeks (Kg) (IQR)	5.4 (4.9-5.9)	5.4 (5.2-5.7)	0.920**
Median weight at 14 weeks (Kg) (IQR)	6.1 (5.7-6.6)	5.7 (5.4-6.4)	0.545**
Median weight at 6 months (Kg) (IQR)	7.6 (7.4-8.8)	6.9 (6.6-7.8)	0.048**
Median weight at 9 months (Kg) (IQR)	8.9 (8.4-9.5)	7.4 (6.4-7.8)	0.007**
Median weight at 1 year (Kg) (IQR)	9.9 (9.1-10.3)	9.7 (8.5-9.8)	0.275**
Breastfed (BF) at birth (%)	100%	79.2%	0.004 [§]
Breastfed (BF) at 6 weeks (%)	93.5%	69.2%	0.022 [§]
Breastfed (BF) at 10 weeks (%)	86.2%	66.7%	0.099 [§]
Breastfed (BF) at 14 weeks (%)	92%	55.6%	0.031 [§]
Breastfed (BF) at 6 months (%)	82.4%	37.5%	0.007 [§]
Breastfed (BF) at 9 months (%)	70.6%	40%	0.077 [§]
Breastfed (BF) at 1 year (%)	83.3%	50.0%	0.099 [§]
Median gestation age in weeks (IQR)	39.3 (38-40)	38.5 (37.8-40.1)	0.349**
Median maternal CD4 count (cells/mL) (IQR)	~	275 (176-381)	
Morbidity (%):			
<u>Birth to 6 weeks</u>			
- Cough/fever	4 (80%)	1 (50%)	0.762 [§]
- Diarrhoea	1 (20%)	0 (0%)	
- Skin rash	0 (0%)	1 (50%)	
<u>6 weeks to 10 weeks</u>			
- Cough/fever	1 (33.3%)	1 (100%)	1.000 [§]
- Diarrhoea	2 (66.7%)	0 (0%)	
- Skin rash	0 (0%)	0 (33.3%)	
<u>10 weeks to 14 weeks</u>			
- Cough/fever	1 (16.7%)	1 (16.7%)	1.000 [§]
- Diarrhoea	4 (66.7%)	4 (66.7%)	
- Skin rash	1 (16.7%)	1 (16.7%)	
<u>14 weeks to 6 months</u>			
- Cough/fever	3 (75%)	1 (100%)	1.000 [§]
- Diarrhoea	1 (25%)	0 (0%)	
- Skin rash	0 (0%)	0 (0%)	
<u>6 months to 9 months</u>			
- Cough/fever	0 (0%)		~
- Diarrhoea	3 (75%)	~	
- Skin rash	1 (25%)		
<u>9 months to 1 year</u>			
- Cough/fever	1 (0%)	2 (100%)	0.238 [§]
- Diarrhoea	2 (75%)	0 (0%)	

2.3 Discussion

In this observational cohort, there were some significant differences between HIV-exposed uninfected infants and their unexposed counterparts. The median birth weight of HIV-exposed uninfected (HEU) infants was significantly lower compared to the HIV-unexposed (HU) infants, as well as some of the time points postnatally. Lower birth weight and poor growth has been described before in previous cohorts (Rollins *et al.*, 2013) (Mussi-pinhata *et al.*, 2006) (C. Epalza *et al.*, 2010) (Kuhn *et al.*, 2006) (Mazzola *et al.*, 2011) (Filteau, 2009) (Jones *et al.*, 2011) (Kidzeru *et al.*, 2014). In a separate cohort, we found some of these differences observed in this study (Kidzeru *et al.*, 2014). We found that HEU infants had a significantly lower birth weight and weight at 6 and 14 weeks of age were significantly lower in the HEU infants compared to their un-exposed counterparts (Kidzeru *et al.*, 2014). However, these differences were not observed at all time points, and the differences disappeared by one year after birth. These differences could be due to the lower proportion of breastfed infants among the HEU.

It has been recommended by WHO that infants in developing countries born to HIV infected mothers be exclusively breastfed, since this improves HIV-free survival (UNAIDS/WHO, 2010) (Yezingane Network and UNICEF, 2011). Exclusive breastfeeding has been shown to be safer compared to mixed feeding for infants born to HIV-positive mothers (Thomas, 2007). There is substantial evidence that HIV infected mothers who exclusively breastfed have a lower risk of transmitting HIV to their infants (Coutsoudis *et al.*, 2001) (Coutsoudis *et al.*, 1999) (Coutsoudis *et al.*, 2001). All HIV infected mothers were on ART and PMTCT and all HEU infants were given nevirapine (NVP) for up to 6 weeks postnatally. All infants regardless of HIV-exposure were breastfed at all-time points except at 6 weeks, 14 weeks and at 6 months postnatally where the frequency of breastfeed HEU infants were significantly lower compared to their unexposed counterparts. In a separate cohort by Kidzeru *et al.*, all HIV infected mothers were on HAART and PMTCT, and also, HEU infants were administered NVP (Kidzeru *et al.*, 2014). We found that the proportion of women who intended to exclusively breastfeed (EBF) infants were significantly lower in the HEU infants compared to their un-exposed counterparts (Kidzeru *et al.*, 2014).

Some of the differences we have observed here could have an effect on vaccine immune responses in these infants. However, we have also earlier shown that after a linear regression estimating the association between HIV exposure and T cell proliferation including other *a priori* confounders, HIV exposure was significantly associated with better CD4⁺ proliferative responses to BCG and staphylococcal enterotoxin B (SEB) after adjusting for birth weight, feeding mode and gestational age (Kidzeru *et al.*, 2014). Also, maternal CD4⁺ cell count was not significantly associated with CD4⁺ or CD8⁺ T cell proliferation. The maternal median duration of exposure to antiretroviral was not a predictive factor of CD4⁺ or CD8⁺ proliferative responses to BCG and SEB (Kidzeru *et al.*, 2014). Therefore, HIV-exposure may play a role amidst the other factors between the groups that could potentially affect MDSC frequency.

Moreover, the main aim of this study is to evaluate the effect of MDSC on immune responses to EPI vaccines in infants and mothers as well as in HEU versus HU infants, and their HIV infected and uninfected mothers. We evaluated the quantity and ontogeny of MDSC, the effect of MDSC on T cell proliferation and the effect of MDSC on cytokine production to vaccines and antigens in infants and mothers. We evaluated these differences generally, and in a sub-analysis between HEU versus HU infants from birth, followed-up at 6, 10 and 14 weeks, at 6 and 9 months and at one year postnatally; and between HIV+ versus HIV- mothers at delivery and at one year postpartum.

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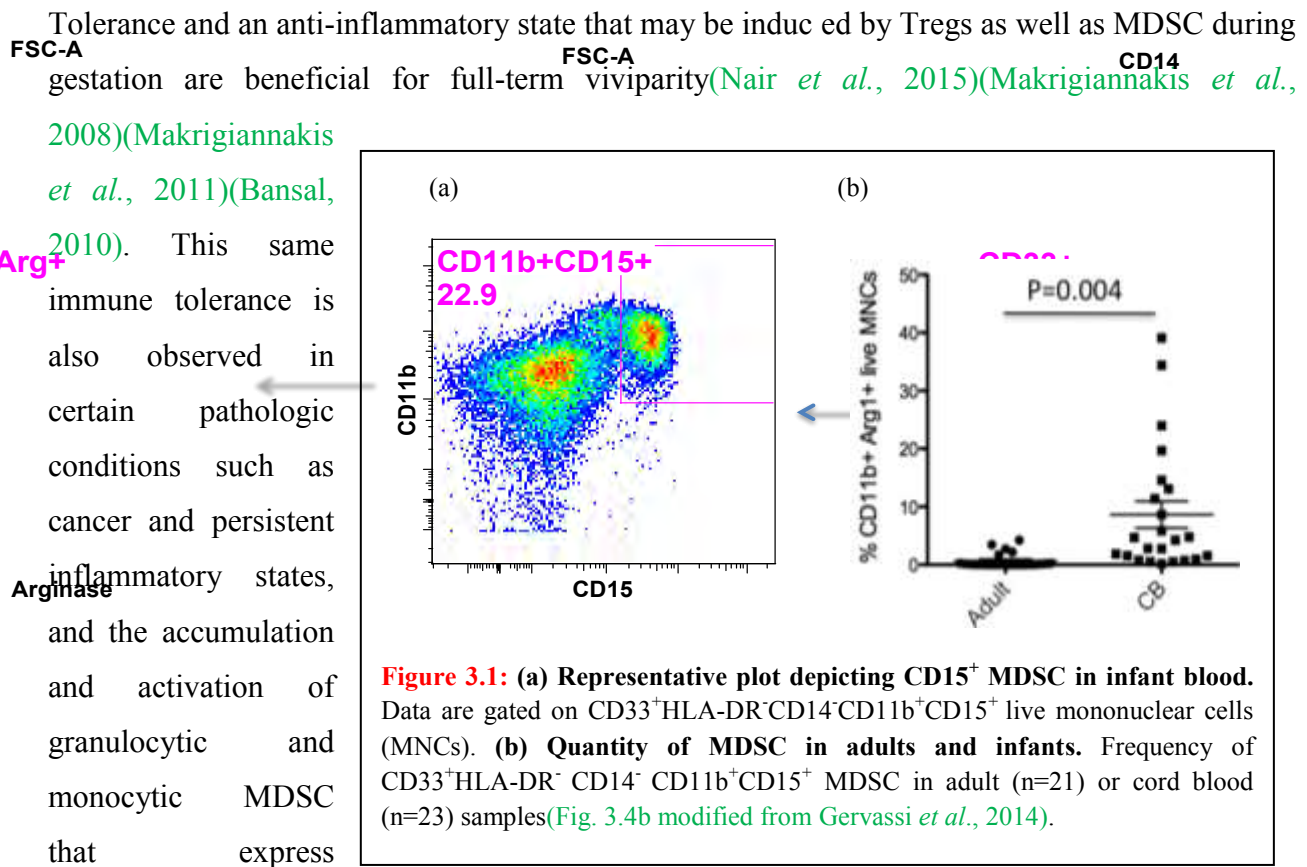
CHAPTER 3

QUANTITY AND ONTOGENY OF MYELOID DERIVED SUPPRESSOR CELLS (MDSC) FROM BIRTH TO ONE YEAR POSTPARTUM

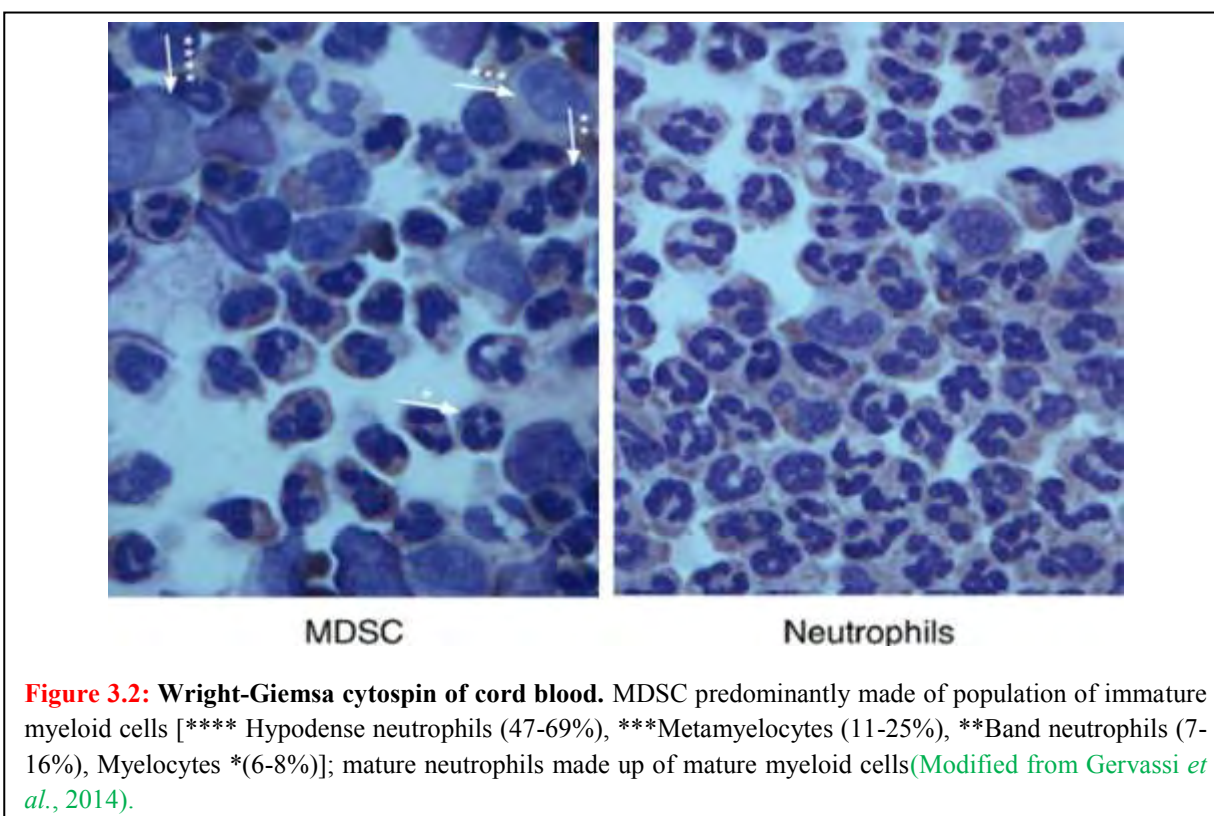
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3.1 Introduction

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of activated immature myeloid cells with suppressive function (Poschke *et al.*, 2012) (Ostrand-Rosenberg *et al.*, 2012) (Gabrilovich *et al.*, 2007). These cells have been well described in cancer patients and very few can be found in healthy adults (Gabrilovich *et al.*, 2012) (Gantt *et al.*, 2014). MDSC can be divided into two different subtypes, either granulocytic or monocytic. In our group, we showed that the frequency of M-MDSC were less than 1% of live cells in healthy individuals, and did not differ significantly between cord blood and adult peripheral blood (A. Gervassi *et al.*, 2014). Granulocytic MDSC are not generally seen at elevated frequencies in healthy adults and have recently been described in healthy infants (Rieber *et al.*, 2013). In our group, the granulocytic MDSC that we observed in cord blood highly expressed CD15 (Gervassi *et al.*, 2014) (Figure 3.1). Hence, throughout the remainder of this thesis we will use MDSC synonymously with G-MDSC.



suppressive factors including Arginase-1 (Arg), reactive oxygen species (ROS), and inducible nitric oxide synthase (NOS) have been found (Poschke *et al.*, 2012)(Gabrilovich *et al.*, 2012)(Ostrand-Rosenberg *et al.*, 2012). Most research on MDSC to date has focused on murine cancer models and in humans with malignancy(Poschke *et al.*, 2012)(Gabrilovich *et al.*, 2012)(Ostrand-Rosenberg *et al.*, 2012). Our group and others have observed high frequency of

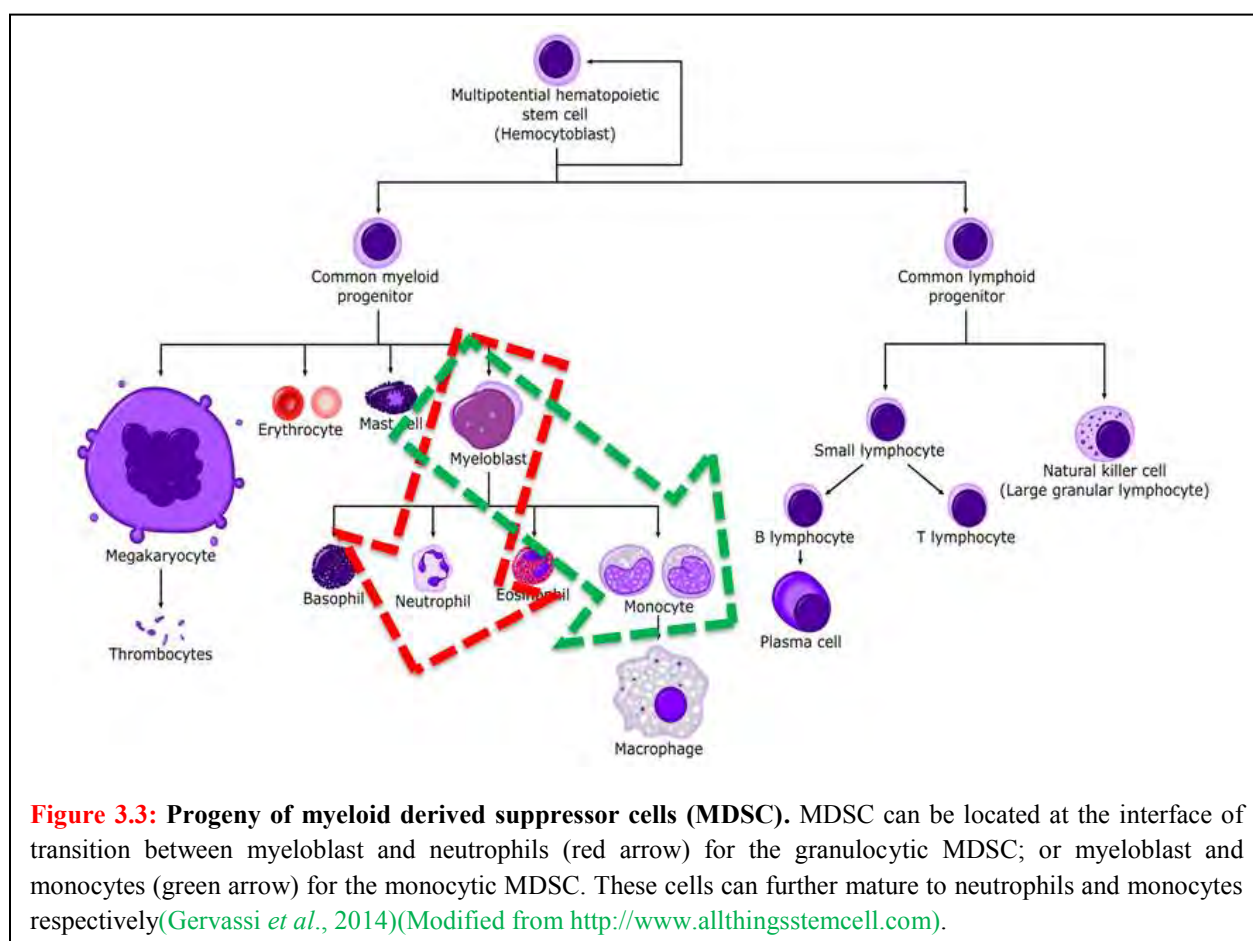


granulocytic (G)-MDSC in cord blood and maternal blood during pregnancy(Rieber *et al.*, 2013)(Gervassi *et al.*, 2014). This environment may persist after birth leading to increased levels of MDSC that may have suppressive effects on the infant's immunity. Using Wright-Giemsa cytospin staining on cord blood mononuclear cells (CBMC) (Figure 3.2), we recently showed that MDSC in infants are predominantly made up of a population of immature myeloid cells distinct from mature neutrophils. MDSC predominantly made up of population of immature myeloid cells with hypodense neutrophils, Metamyelocytes, band neutrophils and myelocytes, whereas mature neutrophils consisting of mature myeloid cells(Gervassi *et al.*, 2014). In the haematopoietic stem cell progeny, MDSC are located at the interface between myeloblast and

monocytes for the monocytic MDSC, or myeloblast and neutrophils for the granulocytic MDSC (Figure 3.3). These cells can either mature to monocytes and neutrophils respectively. In this chapter, we aim to evaluate the quantity and ontogeny of granulocytic MDSC in infants from birth to one year of life and in mothers at delivery and one year postpartum.

3.1.1 Myeloid Derived Suppressor Cells (MDSC) presence and ontogeny

Several studies have measured MDSC by identifying different cellular markers including the expression of CD14 (Monocytic MDSC)(Qin *et al.*, 2013)(Schlecker *et al.*, 2012)(Kotsakis *et al.*, 2012), CD15 (Granulocytic MDSC)(Pillay *et al.*, 2013)(Khaled *et al.*, 2014)(Trellakis *et al.*, 2013)(Rieber *et al.*, 2013)(Kotsakis *et al.*, 2012)(Gervassi *et al.*, 2014) and the potential immuno-suppressor molecule Arginase (Arg) (Greten *et al.*, 2011)(Gervassi *et al.*, 2014).



It has been proposed that the frequency of MDSC in mothers and fetuses is increased during gestation to allow tolerance between the mother and the fetus(Rieber *et al.*, 2013)(Gervassi *et al.*, 2014)(Nair *et al.*, 2014). This has been shown to persist postnatally in infants(Gervassi *et al.*, 2014).

Little is known about the effect of MDSC in HIV-exposed uninfected infants and HIV+ mothers. High MDSC levels have been shown in the peripheral blood mononuclear cells (PBMC) of HIV+ individuals compared to healthy controls(Qin *et al.*, 2013)(Gama *et al.*, 2012)(Vollbrecht *et al.*, 2012). HIV Type 1 gp120 has been shown to induce expansion of MDSC and is suggestive of a typical phenotype that may be linked to viral replication(Garg *et al.*, 2013), and MDSC have been shown to be induced by hepatitis C virus(Tacke *et al.*, 2013). Also, du Plessis *et al.* have shown an increase in MDSC in TB disease(du Plessis *et al.*, 2013). In this chapter the effect of HIV-exposure on MDSC frequency in uninfected infants and HIV-infection in mothers was also assessed.

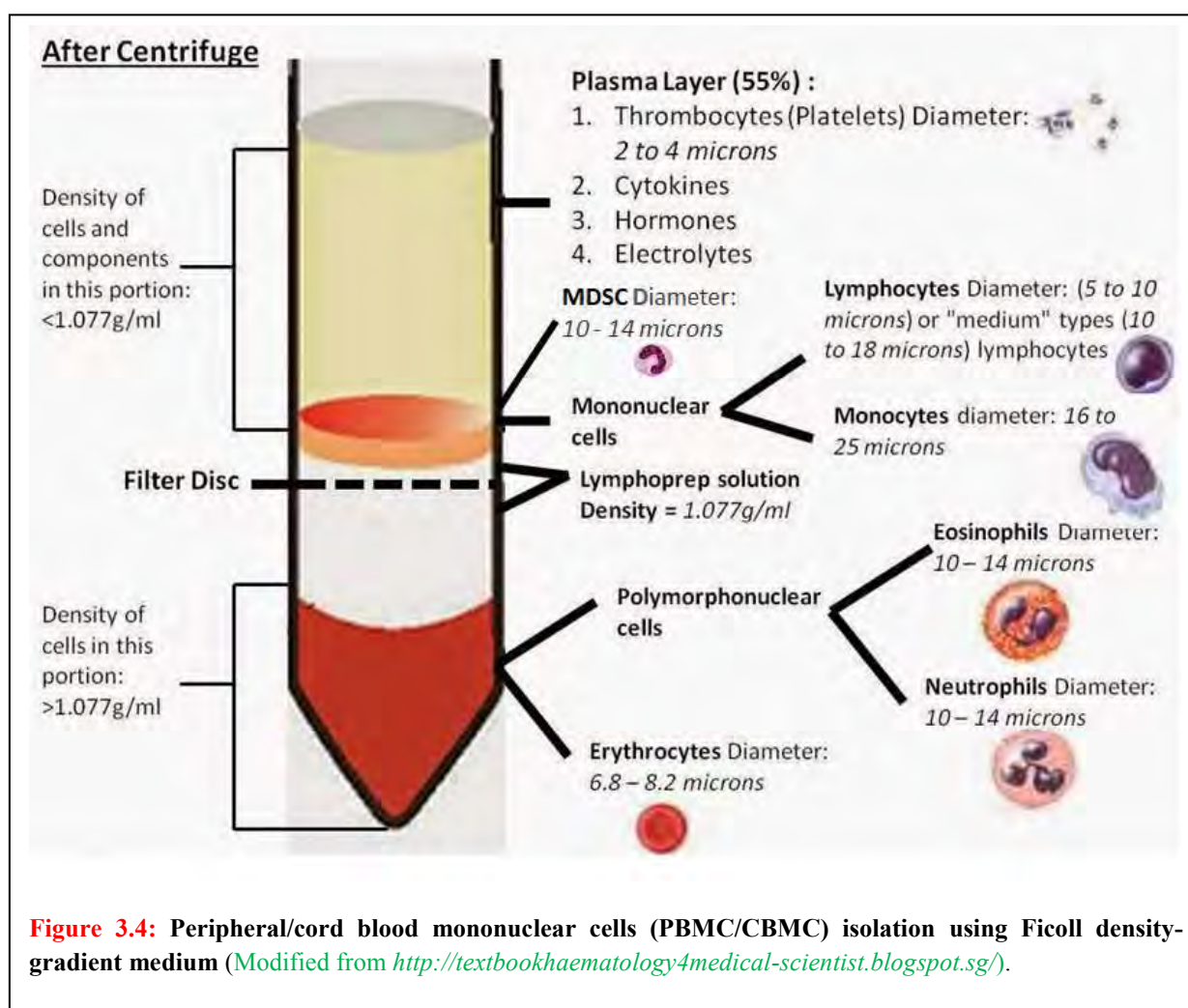
Hence, in this chapter we hypothesize that in infants and mothers the MDSC frequency will decrease with age/time postpartum and that MDSC frequency in HIV-exposed uninfected (HEU) infants and HIV-infected (HIV+) mothers will be higher compared to the HIV un-exposed (HU) infants and HIV-uninfected (HIV-) mothers respectively.

It must be emphasised that, the inclusion of mothers in this study is to test our hypothesis that MDSC are present during gestation and wane afterwards and mothers in this case are not a true representative of normal adults since they have just been pregnant. Also, mothers would have to tolerate their fetus as they may have HLA mismatch of paternal origin.

3.2 Materials and method

The primary outcome measure in this chapter was the frequency of granulocytic MDSC in PBMC quantified by the expression of $HLADR^+CD14^+CD33^+CD11b^+CD15^+$ cells (see Figure 3.5). The overall process of quantifying MDSC involved three main procedures:

1. Peripheral/cord blood mononuclear cells (PBMC/CBMC) isolation
2. Glycophorin A micro beads red blood cell (RBC) depletion technique
3. Flow cytometry [MDSC cellular staining characterisation]



3.2.1 Peripheral/cord blood mononuclear cells (PBMC/CBMC) isolation

Between 1-5mL whole/cord blood was collected from infants at birth, 6, 10 and 14 weeks; at 6 and 9 months and one year of age. From mothers, about 10mL of whole blood was collected at time of delivery and again followed-up at one year postpartum. All blood samples were collected in heparinised tubes and transported to the laboratory within 6 hours of blood draw. Samples were processed immediately after they were delivered to the lab from the collection site. Plasma was collected for storage at -80°C and the whole blood diluted with phosphate buffered saline (PBS) for mononuclear cells isolation.

As previously described(Gervassi *et al.*, 2014), we used a sterile 15mL polystyrene tube containing 4mL Ficoll density-gradient separation medium per 10mL of diluted whole blood. The PBS-diluted whole blood was poured gently with the tube tilted at an angle of 45° to prevent any mixing with the separation medium. After prolonged centrifugation at 2500 rpm, the 15mL polystyrene tube was used to “purify” and isolate the PBMC. Ficoll medium is a specially formalized separation medium that has a density of 1.077g/mL. Since RBCs and polymorphonuclear granulocytes (Mature neutrophils, Eosinophils) are cells that have a density more than 1.077g/mL, moved to the pellet at the bottom of the tube(Breit, 2013). As a consequence, the Ficoll medium is displaced and pushed upwards. The PBMC/CBMC – lymphocytes and monocytes as well as MDSC (immature myeloid cells) will concentrate at the interface between the upper phase (plasma, thrombocytes) and the lower phase (Ficoll) according to their specific density with density lighter than 1.077g/mL moved above the Ficoll density-gradient medium, and this layer is commonly known as the buffy coat layer(Breit, 2013) (Figure 3.4). In some infants both CBMC and PBMC were obtained and frequency of MDSC compared.

3.2.2 Glycophorin A micro beads red blood cell (RBC) depletion technique

In certain samples where the intensity of PBMC/CBMC contamination by red blood cells (RBC) was intense, RBC was depleted using glycophorin A micro beads (Miltenyi Biotech). Briefly, where glycophorin A micro beads RBC depletion technique was required, it was performed as follows: in a 5mL FACS tube, cells were suspended in ice cold MACS separation buffer [0.1% heat inactivated human serum (HS) and 0.4% of 2mM EDTA, into 500mL phosphate buffered saline (PBS)] i.e. 16uL MACS buffer per 1×10^7 cells, plus 4uL glycophorin A micro beads. The

cell suspension was further incubated in the dark at 4°C for 15 minutes. After, the suspension was then topped-up to 2.5mL with MACS separation buffer and the tube inserted in the EasySep[®] magnet for 10 minutes, without a cap. Positively selected RBC bound to the walls of the tube, and the rest of the cells remained in the cell suspension. After incubation, the negatively selected cells were decanted out of the tube with the tube still in the magnet (see Figure 4.1). The depletion process was repeated twice, to complete three rounds of RBC depletion.

3.2.3 Flow cytometry [Characterization of MDSC by cellular staining]

Briefly, using a pre-labelled 96 well round bottom plate, about 1×10^6 cells/well of the different cell populations i.e. whole CBMC and/or PBMC, were plated for staining. Staining for MDSC quantification was done only on fresh blood samples, and not cryopreserved, as it has been shown that few MDSC survive cryopreservation (Kotsakis *et al.*, 2012). Extracellular staining with 50uL of optimized concentration of flourochrome-bound antibodies and Violet fluorescent

reactive dye (VIVID) to stain dead cells was performed (Table 3.1). Cells were incubated for 20 minutes in the dark at RT. After incubation, the

Table 3.1: MDSC multicolour flow cytometry panel. The seven colour flow cytometry panel employed with the various antibodies markers, fluorochromes, their suppliers and rationale (importance for inclusion in the panel).

Antibody	Fluorophore	Clone	Supplier	Rationale
VIVID	Pacific Blue	-	Life technologies	Dead cells exclusion
CD11b	PE-Cy7	ICRF44	BD Biosciences	Leukocytes
CD14	PE	M5E2	BD Biosciences	Monocytes
CD15	APC	H198	Biolegend	Neutrophils
CD33	PE-Cy5	WM53	BD Biosciences	Myeloid cells
HLA-DR	APC-Cy7	L243	BD Biosciences	Macrophages, B-cells and DC
Arginase 1	Alexa Flour 488	6G3	Hycult biotech/ Invitrogen	Potential suppressor molecule

cells were washed off the excess VIVID with 200uL of 2% FACS wash buffer i.e. 2% Human Serum in PBS, and the plate was centrifuged at 2100 rpm, repeated once. Cells were further fixed with 100uL of 10% BD FACS[™] Lysing Solution in distil H₂O to lyse any red cells and fix white cells, and incubated for 10 minutes. After incubation, the cells were washed, with 200uL of 2% FACS wash buffer, repeated once.

Further, using 200uL of 10% BD FACS[™] Permeabilizing Solution 2 in distilled H₂O; cells were permeabilized, and incubated for another 10 minutes. After incubation, the plate was

centrifuge to take off the permeabilizing solution 2, and the cells were washed with 200uL of 2% FACS wash buffer, repeated once. The plate was now ready for intracellular staining with an optimized concentration of fluorochrome conjugated Arginase 1 antibody. Acquisition was performed using BD LSRII flow cytometer and analysis with FlowJo v9.4.7. **Table 3.1** shows the multicolour flow cytometry panel used.

Granulocytic MDSC was defined as $CD33^+$, $HLA-DR^-$, $CD14^-$, $CD11b^+$ and $CD15^+$ immature myeloid cells as the cells of interest. Flow cytometry was used to assess all the cell populations, and we also evaluated the presence of MDSC after HIV exposure; between HIV-exposed uninfected (HEU) and HIV unexposed (HU) infants from birth through one year postnatal, and between HIV-infected (HIV+) and HIV uninfected (HIV-) mothers at time of delivery and a year postpartum. MDSC were reported as frequency of Live cells, and absolute numbers were calculated as number of cells per millilitre of suspension media i.e. 1% formaldehyde.

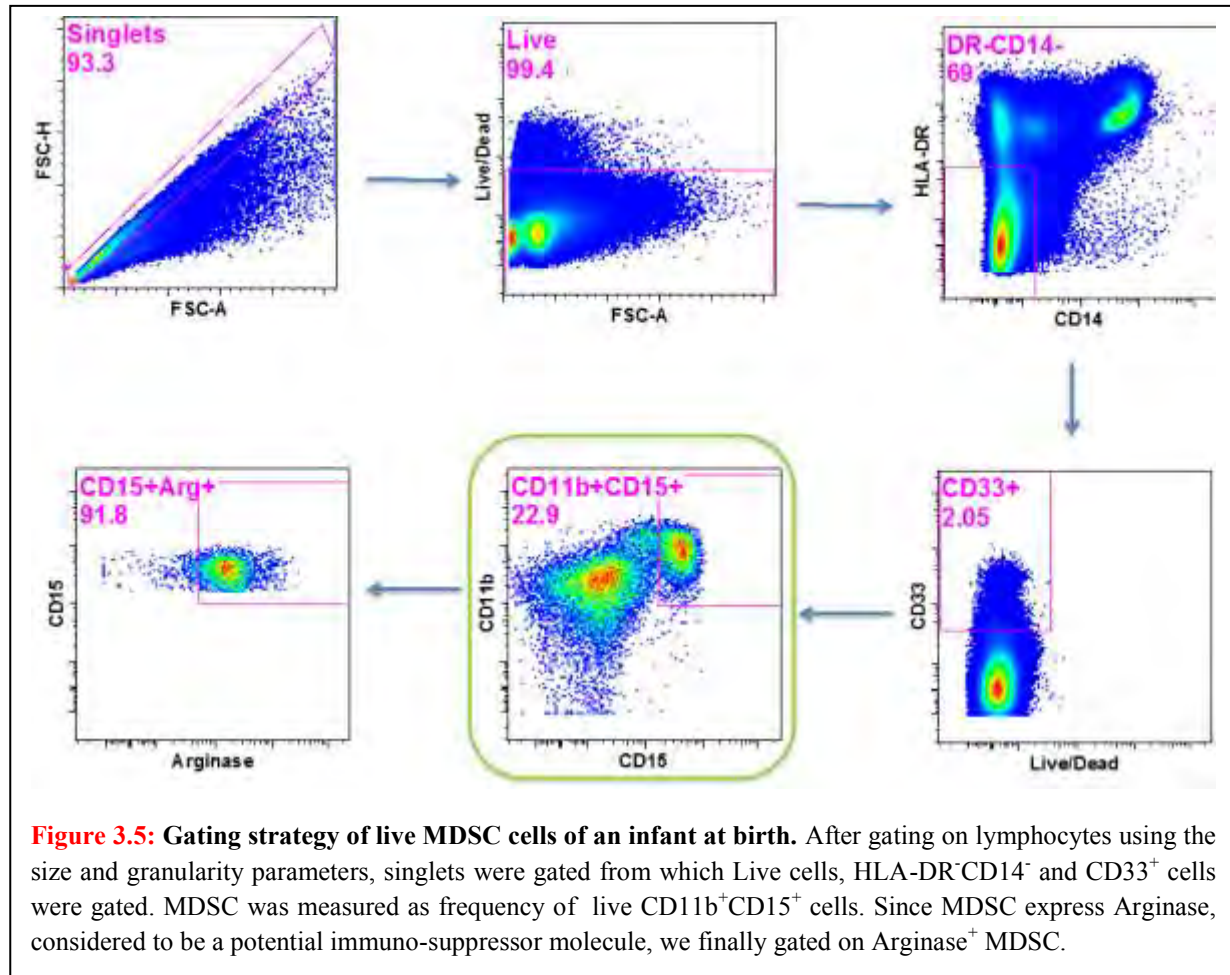
3.2.3.1 Data analysis

Flow cytometry data was extracted for analysis after sample acquisition. Below is a summary of the data analysis strategy.

After sample acquisition by flow cytometry, the flow files were first analyzed using FlowJo v9.4.7 (Tree Star). After compensation was performed using singly stained compensation controls with the seven fluorochromes employed (**see table 3.1**), gating for singlets (using the size and granularity parameters) and live cells was performed. Using Fluorescence Minus One (FMO's) to all the fluorochromes used to assign the gates of interest, $HLA-DR^-$ and $CD14^-$ cells were selected, from which $CD33^+$ cells were gated. Finally, $CD15^+$ cells were gated against $CD11b^+$ cells, (**Figure 3.5**) and (**Table 3.1**). ***Data were reported as frequency of live $CD11b^+CD15^+$ expressing cells.*** Although Arg staining was performed, it was not used to define MDSC, as it is not conclusively known whether all MDSC suppression is mediated through this mechanism. However, routinely >90% cells were Arg positive.

We obtained full blood counts (FBC) from infants at birth and at 6 months of age using the Sysmex® XN-Series automated haematology analyser. Correlations were performed between MDSC versus neutrophil frequencies and counts at birth and later at 6 months of life. MDSC

absolute counts were obtained as number of $CD11b^+CD15^+$ cells per 10^6 PBMC in a millilitre of suspension media i.e. 1% formaldehyde. However, as one of the limitations of the study, we did not take into consideration the complete blood count when calculating the MDSC absolute count, and the analysis does not total give a true reflection of the absolute count.



3.2.3.2 Statistical considerations

Different statistical packages were used to complete the data analysis after obtaining data from flow cytometry as listed in the table below (Table 3.2).

Table 3.2: Statistical packages used for data analysis

N°	SOFTWARE PROGRAM	ANALYSIS TYPE	DATA TYPE
1	FLOWJO v9.4.7 (for MAC, Stanford University, 1995-96; Tree Star, Inc. 1997-2012)	To analyze flow cytometry data after acquisition	Flow cytometry data analysis
2	Microsoft Excel 2013	To clean and organize data	
3	GraphPad Prism v5 (for Windows, GraphPad Software, San Diego California USA, www.graphpad.com”).	To analyze quantity and ontogeny of MDSC from birth to one year of age (infants), and between delivery and one year postpartum (mothers)	Graphics -(MDSC quantity) -(HEU vs. HU) -(HIV+ vs. HIV-)
4	STATA v11 (for windows, StataCorp LP, College station, TX77845, USA).	Statistical tests for cohort description	-Chi-square test -Fisher’s exact test -Wilcoxon rank sum test (Mann-Whitney test)

3.2.3.3 Statistical data analyses

Data analysis was performed by applying the following statistical tests;

Table 3.3: Statistical tests used to complete data analyses

Statistical test	Rationale	Statistical package	Data type
Chi-square test	Relationship between two categorical variables	STATA v11	Cohort description (HEU vs. HU)
Fisher’s exact test	Relationship between two categorical variables (one or more of the values in the cells has an expected frequency ≤ 5)	STATA v11	Cohort description (HEU vs. HU)
Wilcoxon rank sum test (Mann-Whitney test)	Compare continuous data between two categories that is not normally distributed.	- GraphPad Prism v5 - STATA v11	- (HEU vs. HU) - (MDSC ontogeny)
Wilcoxon matched-pairs sign rank test	Compare continuous data between two matched categories that is not normally distributed.	- GraphPad Prism v5	-Quantity of MDSC (HEU vs. HU) (HIV+ vs. HIV-)
Spearman Correlation	Correlations between two continuous variables	- GraphPad Prism v5	(Infants MDSC frequencies vs. Maternal MDSC frequencies) ect.

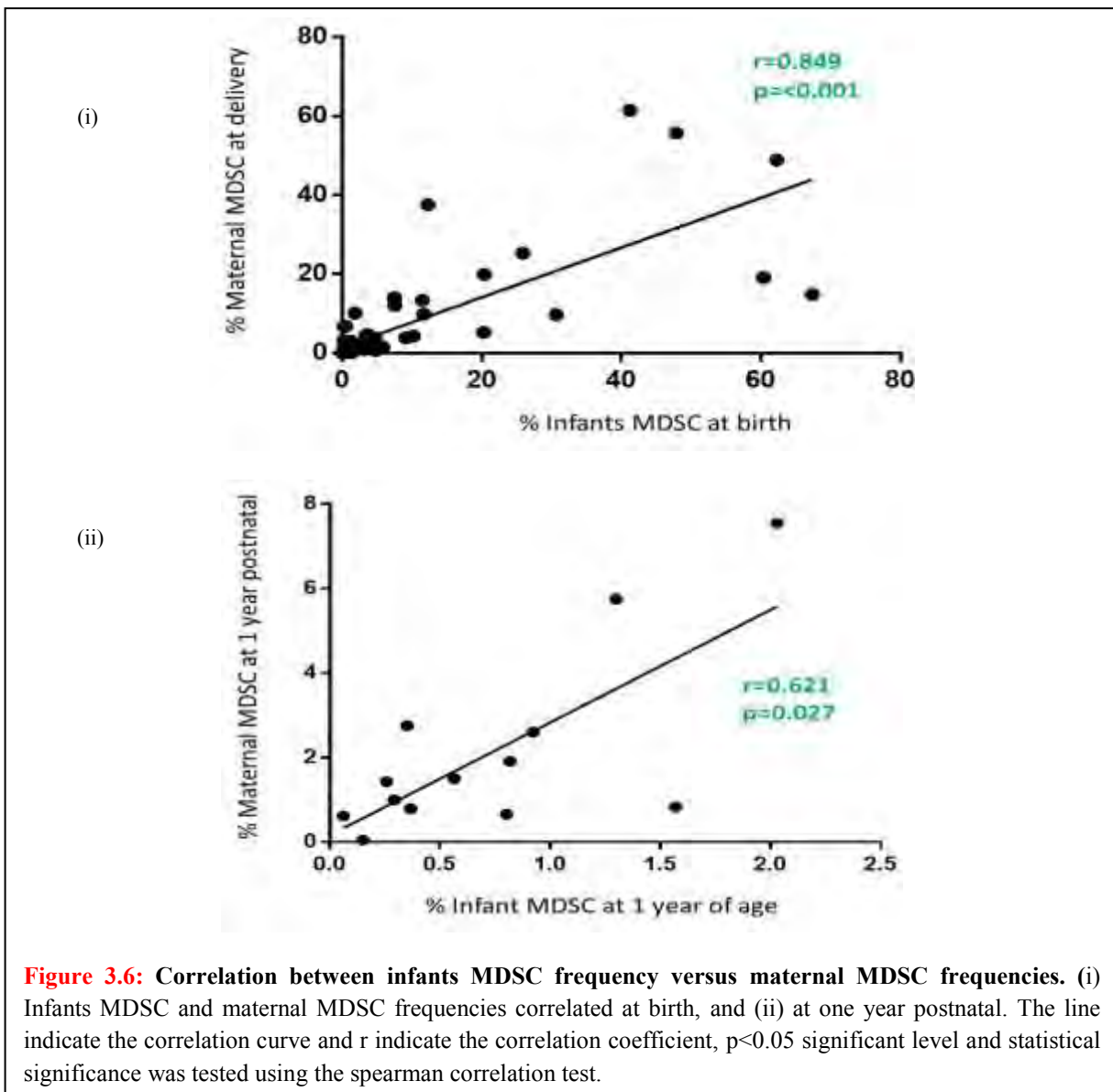
3.3 Results

In this chapter we were interested in quantifying MDSC in infants and mothers from the time of delivery to one year postpartum. We evaluated the presence and ontogeny of granulocytic MDSC as described in [section 3.1.1](#).

To effectively evaluate and understand the ontogeny of MDSC, we investigated whether there was a correlation between MDSC and neutrophil absolute counts and frequencies, since granulocytic MDSC are predominantly immature neutrophils. We obtained full blood counts (FBC) from infants at birth and at 6 months of age. To also understand the relation between infants and maternal MDSC we performed correlations between infants and maternal MDSC frequencies and absolute counts. From the cohort characteristics ([Chapter 2](#)), we obtained infants weights at the follow-up time points as well as gestational ages. We investigated the correlation between infants MDSC frequency and absolute counts versus infants weights at all the follow-up time points at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age. Further, we performed correlations between infants MDSC at all the follow-up time points with the gestational ages.

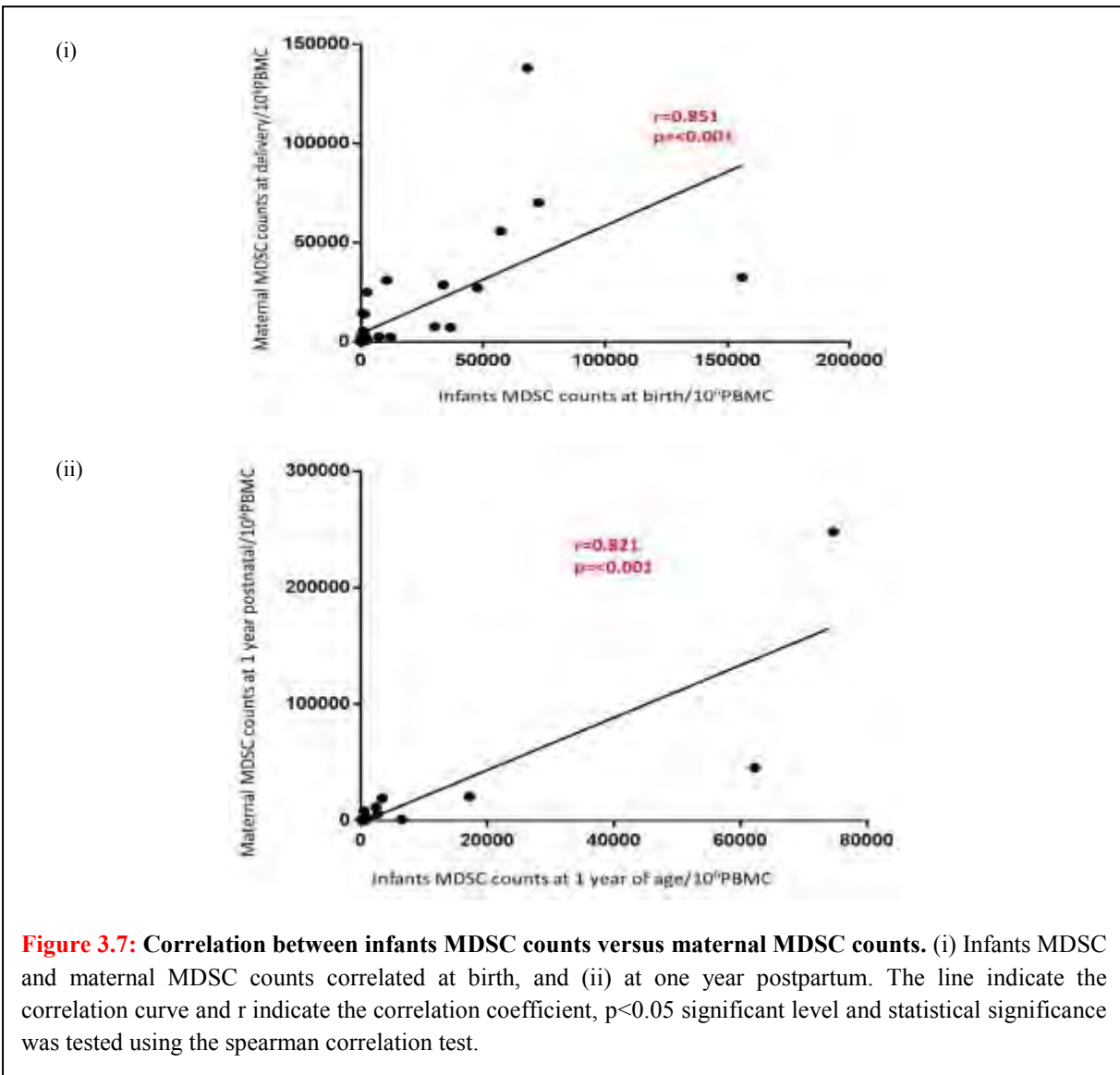
(a) Correlation between the infants MDSC frequencies versus maternal MDSC frequencies

Spearman correlations were performed between infants and maternal MDSC frequencies at delivery and later at one year of age [Figures 3.6 (i and ii)]. At delivery, there was a significant positive correlation between the infants versus maternal MDSC frequencies ($r=0.849$ and $p\text{-value}<0.001$) [Figure 3.6 (i)]. At one year postpartum, the significant positive correlation remained ($r=0.621$ and $p\text{-value}=0.027$) [Figure 3.6 (ii)].



(b) Correlation between the counts of infants MDSC versus maternal MDSC counts

Spearman correlations were performed between infants and maternal MDSC absolute counts at delivery and later at one year of age [Figures 3.7 (i and ii)]. At delivery, there was a positive correlation between infants and maternal MDSC absolute counts ($r=0.851$ and $p\text{-value}=\leq 0.001$) [Figure 3.7 (i)]. This significant positive correlation was also observed at one year postpartum ($r=0.821$ and $p\text{-value}=\leq 0.001$) [Figure 3.7 (ii)].



As shown in [Appendix 3.1 \(Figures A1 and B1\)](#), Spearman correlations were performed between MDSC versus neutrophil frequencies and absolute counts at birth and later at 6 months of life. At birth, there was a significant positive correlation between MDSC frequency of live cells in PBMC versus frequency of leukocytes that were neutrophils in the FBC ($r=0.449$ and $p\text{-value}=0.036$) [[Figure A1\(i\)](#)], but at 6 months of age, there was no correlation ($r=-0.361$ and $p\text{-value}=0.205$) [[Figure A1\(ii\)](#)]. Also, at birth and 6 months of age, there were no correlations between the absolute counts of MDSC versus absolute counts of neutrophils ($r=0.311$ and $p\text{-value}=0.182$) [[Figure B1\(i\)](#)], and ($r=-0.383$ and $p\text{-value}=0.313$) [[Figure B1\(ii\)](#)] respectively.

Also, Spearman correlations were performed between infant MDSC frequencies and absolute counts versus infants' weights at birth, at 6, 10, and 14 weeks, at 6 and 9 months, and at one year of age (see [Appendix 3.1, Figures A2 and B2](#)). There was no significant correlation between the infants' MDSC frequencies and their birth weights ($r=0.071$ and $p\text{-value}=0.560$) [[Figure A2\(i\)](#)]. This lack of significant correlation was also observed at other time points at 6, 10 and 14 weeks ($r=-0.034$ and $p\text{-value}=0.838$; $r=0.099$ and $p\text{-value}=0.599$; and $r=-0.102$ and $p\text{-value}=0.590$ respectively), at 6 and 9 months ($r=0.275$ and $p\text{-value}=0.241$; and $r=-0.038$ and $p\text{-value}=0.889$ respectively), and at one year of age ($r=0.125$ and $p\text{-value}=0.671$) [[Figure A2\(ii-vii\)](#)]. Also, at birth, there was no significant correlation between infant MDSC absolute counts versus birth weights ($r=0.221$ and $p\text{-value}=0.135$) [[Figure B2\(i\)](#)]. This lack of significant correlation persisted at other time points between infants MDSC absolute counts and infants weights at 6, 10 and 14 weeks ($r=0.230$ and $p\text{-value}=0.154$; $r=-0.031$ and $p\text{-value}=0.863$; and $r=-0.072$ and $p\text{-value}=0.701$), at 6 and 9 months ($r=0.141$ and $p\text{-value}=0.512$; and $r=-0.017$ and $p\text{-value}=0.945$), and at one year of age ($r=0.066$ and $p\text{-value}=0.835$) [[Figure B2\(ii\)](#)].

Spearman correlations were also performed between infants MDSC frequencies and absolute counts at birth, at 6, 10, and 14 weeks, at 6 and 9 months, and at one year of age versus infant gestational age (see [Appendix 3.1, Figures A3 and B3](#)). At birth, there was no significant correlation between the infants' MDSC frequencies and gestational ages ($r=0.046$ and $p\text{-value}=0.708$) [[Figure A3\(i\)](#)]. This lack of correlation persisted at 6, 10 and 14 weeks ($r=0.055$

and $p\text{-value}=0.745$; $r=-0.021$ and $p\text{-value}=0.913$; and $r=-0.026$ and $p\text{-value}=0.892$ respectively), at 6 and 9 months ($r=-0.192$ and $p\text{-value}=0.458$; and $r=-0.051$ and $p\text{-value}=0.819$ respectively), and at one year of age ($r=0.433$ and $p\text{-value}=0.141$) [Figure A3(ii-vii)]. Also, at birth, there was no significant correlation between infants' MDSC absolute counts versus gestational ages ($r=-0.143$ and $p\text{-value}=0.345$) [Figure B3(i)]. This lack of correlation persisted at other time points ($r=0.173$ and $p\text{-value}=0.293$ at 6 weeks; $r=-0.174$ and $p\text{-value}=0.326$ at 10 weeks; and $r=-0.168$ and $p\text{-value}=0.374$ at 14 weeks.), at 6 and 9 months ($r=0.272$ and $p\text{-value}=0.210$; and $r=-0.015$ and $p\text{-value}=0.956$), and at one year of age ($r=0.067$ and $p\text{-value}=0.837$) [Figure B3(ii-vii)].

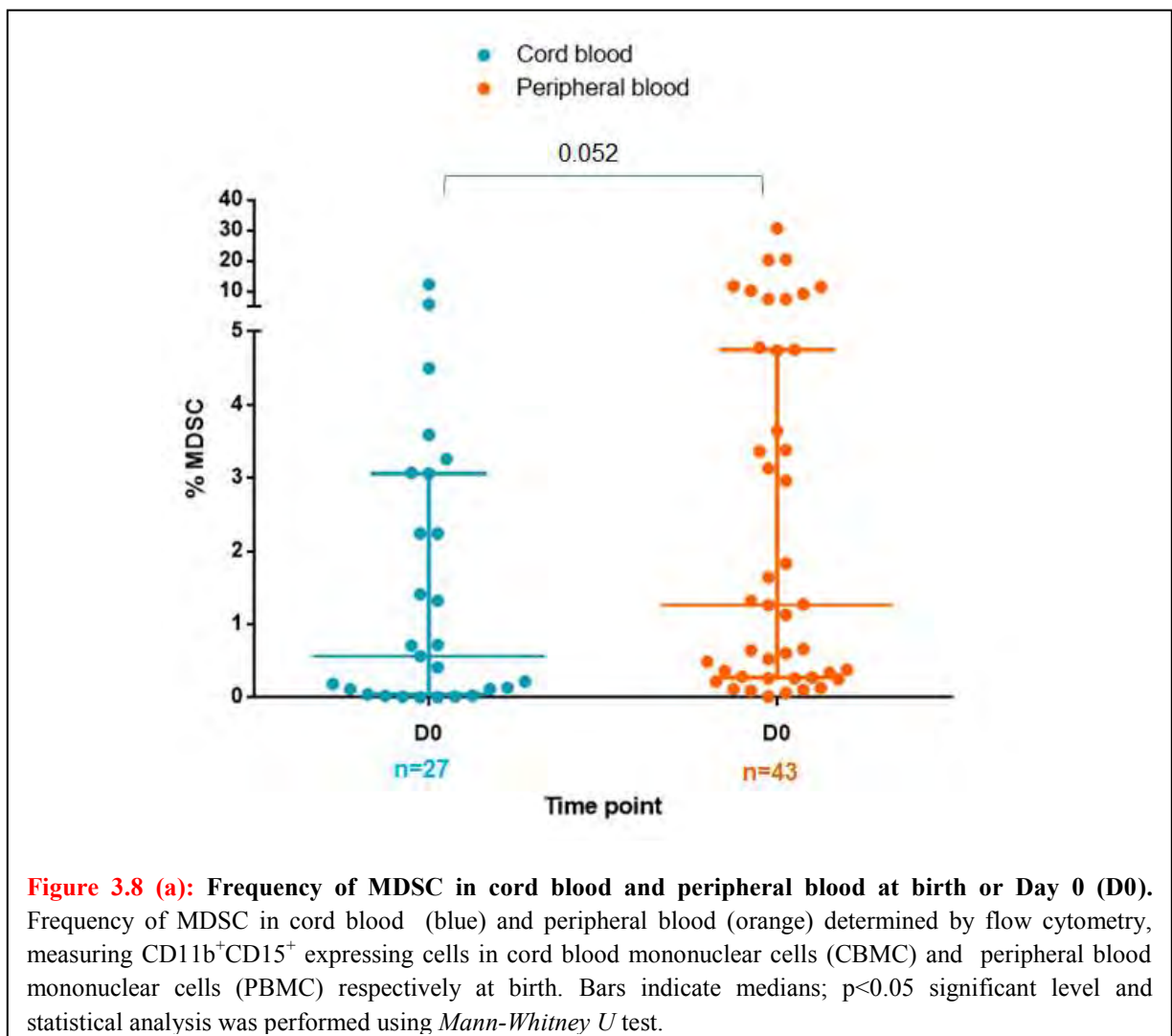
Hence, there was no major correlation between MDSC and neutrophil frequencies or absolute counts at 6 months of age in infants; while at birth the proportion of MDSC increased with increase in the proportion of neutrophils but not the cell counts.

3.3.1 MDSC presence and ontogeny in infants

We evaluated the quantity and ontogeny of MDSC in infants over time from birth to one year of age. This was done by measuring the frequency of $CD11b^+CD15^+$ cells in CBMC/PBMC from fresh blood as described in the methods (see section 3.2).

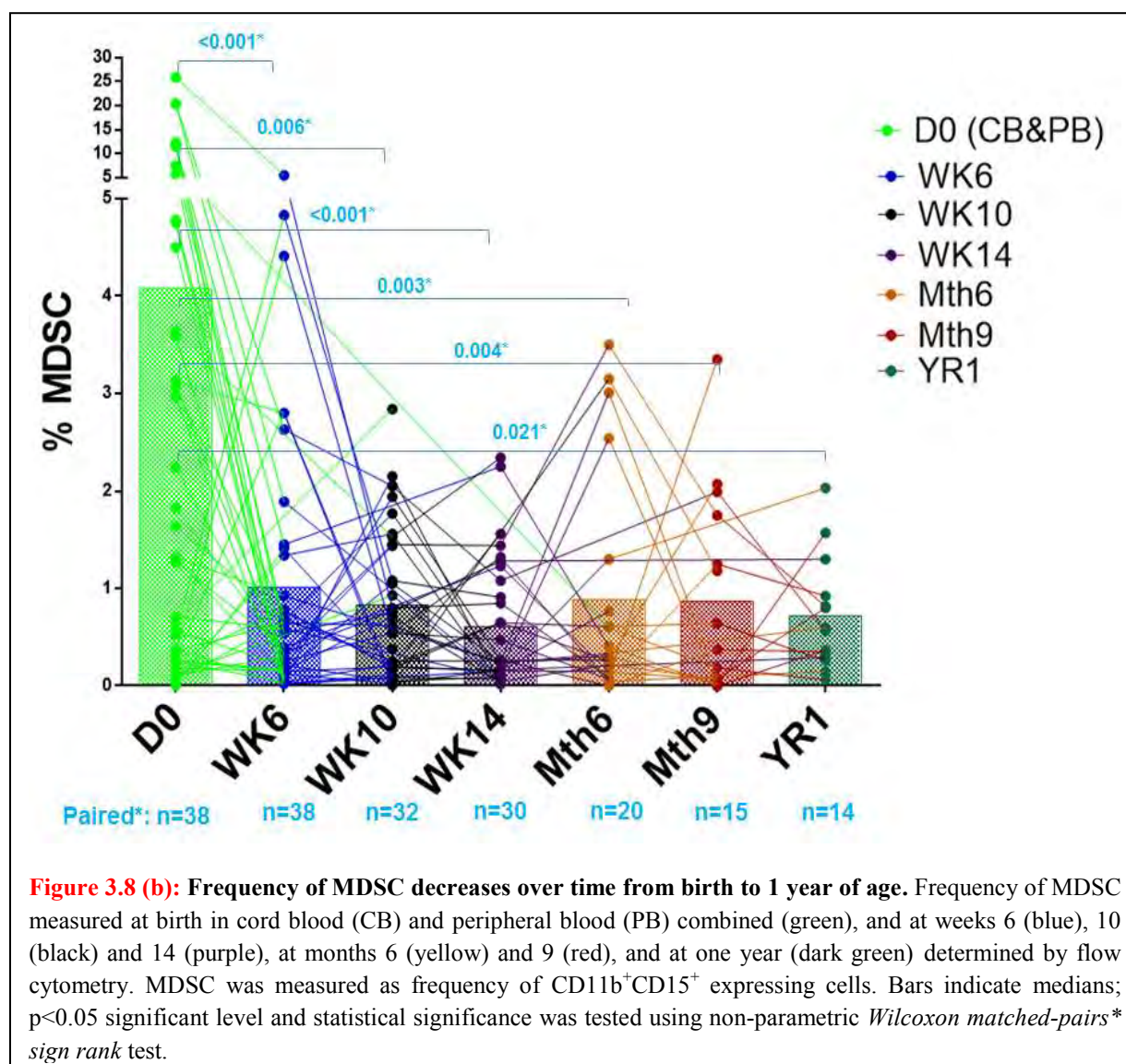
3.3.1.1 Neonatal MDSC frequency in cord blood and peripheral blood at birth

The frequency of MDSC in cord blood (CB) was slightly lower than that of peripheral blood (PB) of neonates at birth (D0) but this difference was not significant (median MDSC in CB=0.564% versus PB=1.260% and p-value=0.052) [Figure 3.8(a)].

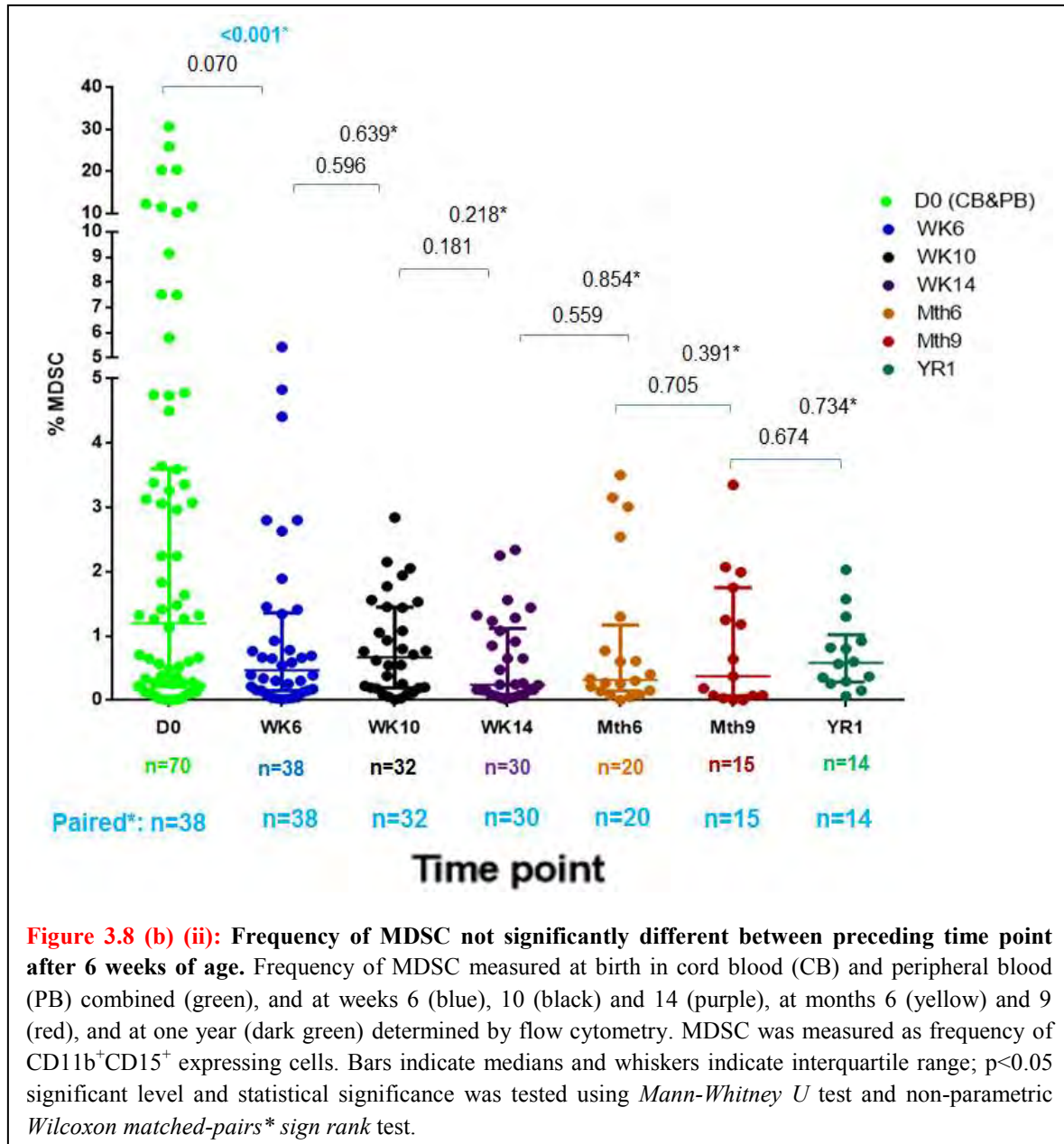


3.3.1.2 Quantity and ontogeny of MDSC over time from birth to one year of age

At birth, there was a significantly higher MDSC frequency compared to other time points. The median MDSC at birth (D0) (cord and peripheral blood combined) was 1.480% versus 0.468% at week 6 ($p<0.001$), 0.664% at week 10 ($p=0.006$), 0.243% at week 14 ($p<0.001$), 0.318% at 6 months ($p=0.003$), 0.371% at 9 months ($p=0.004$) and 0.581% at one year of age ($p=0.021$) [Figure 3.8(b)].



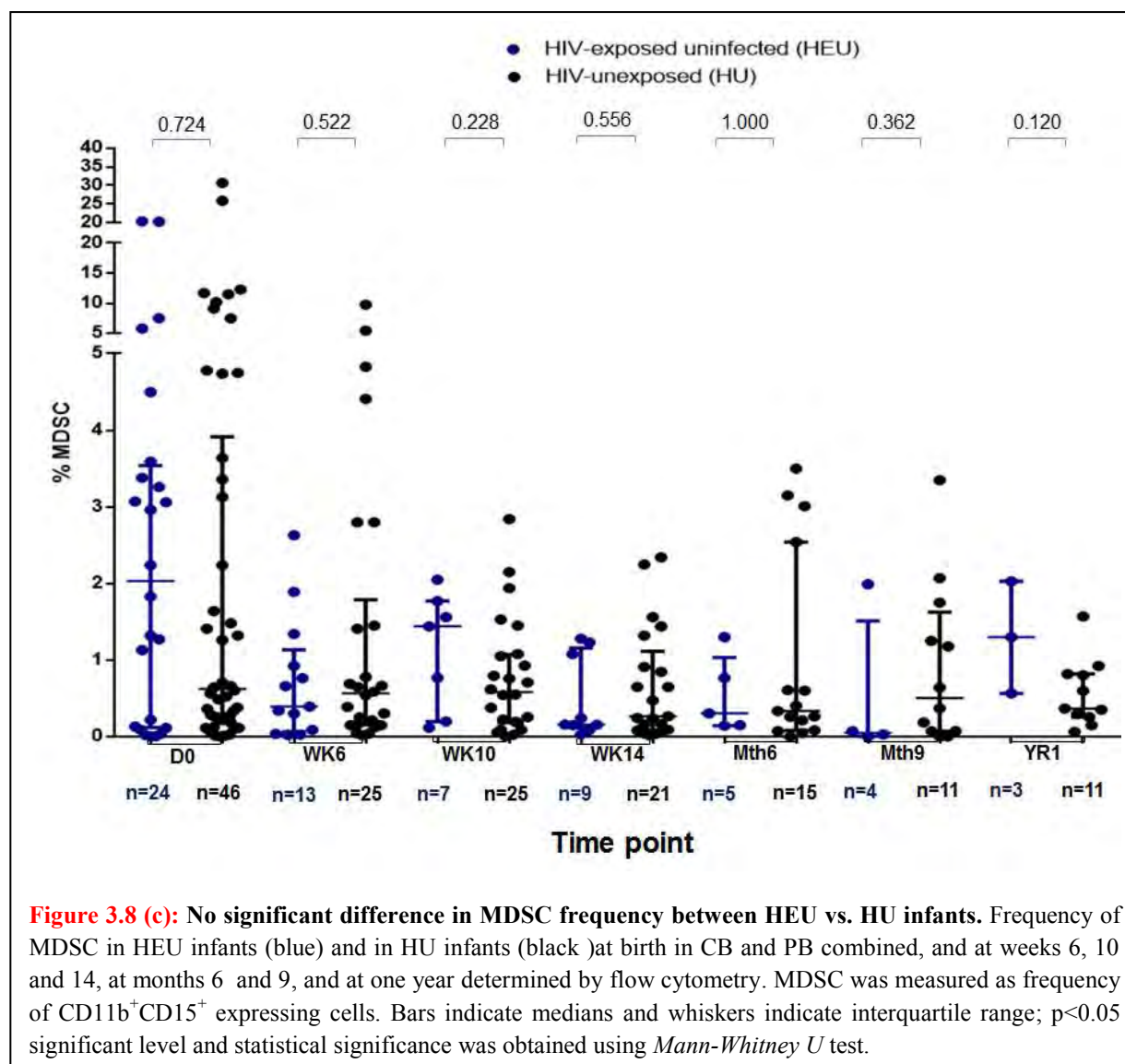
However, between preceding time points from 6 weeks of age, MDSC frequency did not significantly differ between WK6=0.542% versus WK10=0.548% and p-value=0.639, WK10=0.739% versus WK14=0.255% and p-value=0.218, WK14=0.265% versus Mth6=0.303% and p-value=0.854, Mth6=0.354% versus Mth9=0.278% and p-value=0.391, and Mth9=0.371% versus YR1=0.565% and p-value=0.734 [Figure 3.8 (b) (ii)].

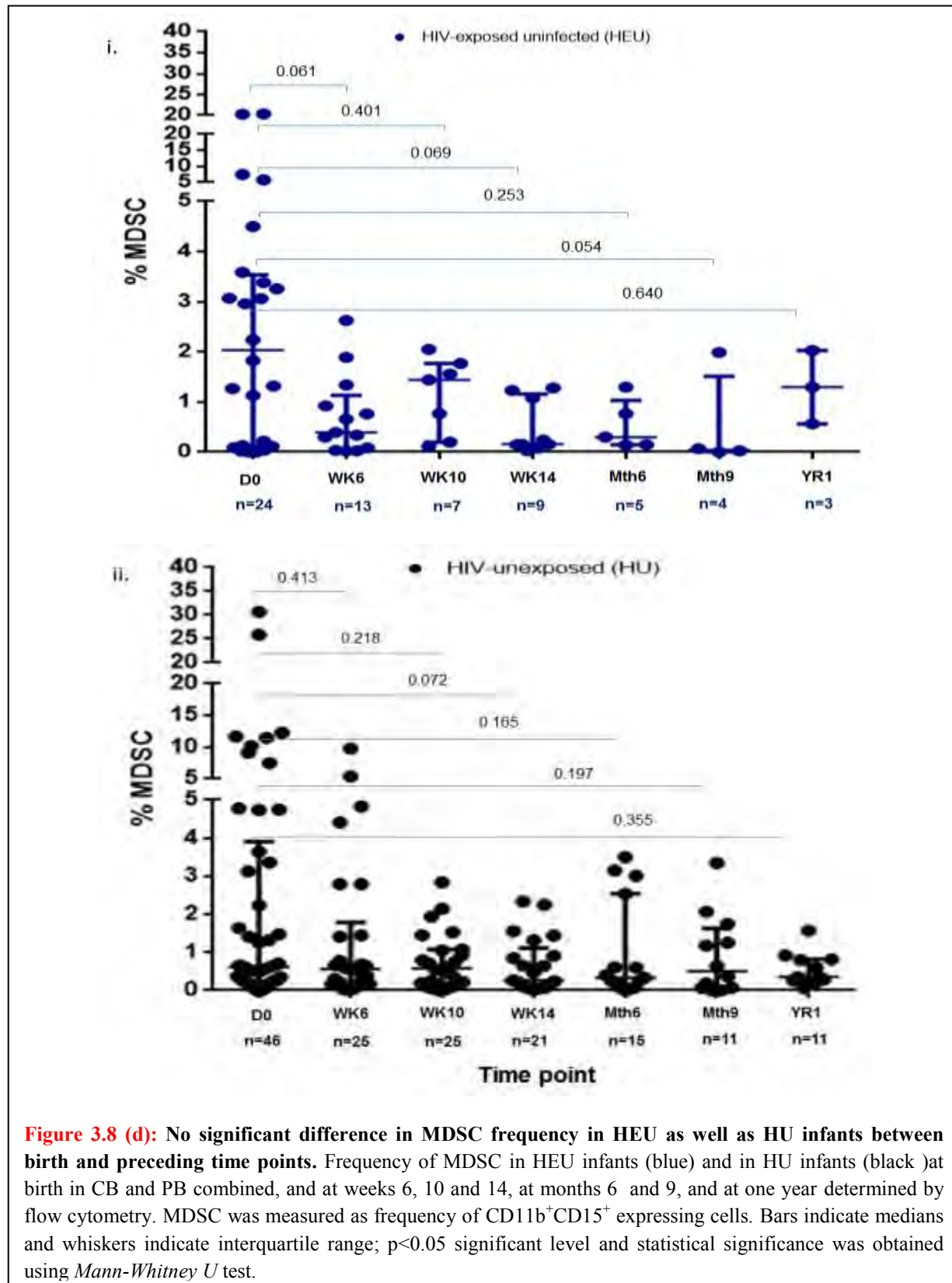


3.3.1.3 MDSC presence and ontogeny in HEU compared to HU infants

We compared the quantity and ontogeny of MDSC in infants born to HIV+ mothers compared to their HIV un-exposed counterparts at each time point.

Although at most time points, MDSC frequency was higher in HEU versus HU infants [Figure 3.8 (c)], significant differences in MDSC frequency in HEU vs. HU infants were not evident [Figure 3.6 (c)]. Also, there was no significant difference between MDSC frequency at birth versus preceding time points in both HEU as well as HU infants [Figure 3.6 (d)(i-ii)].



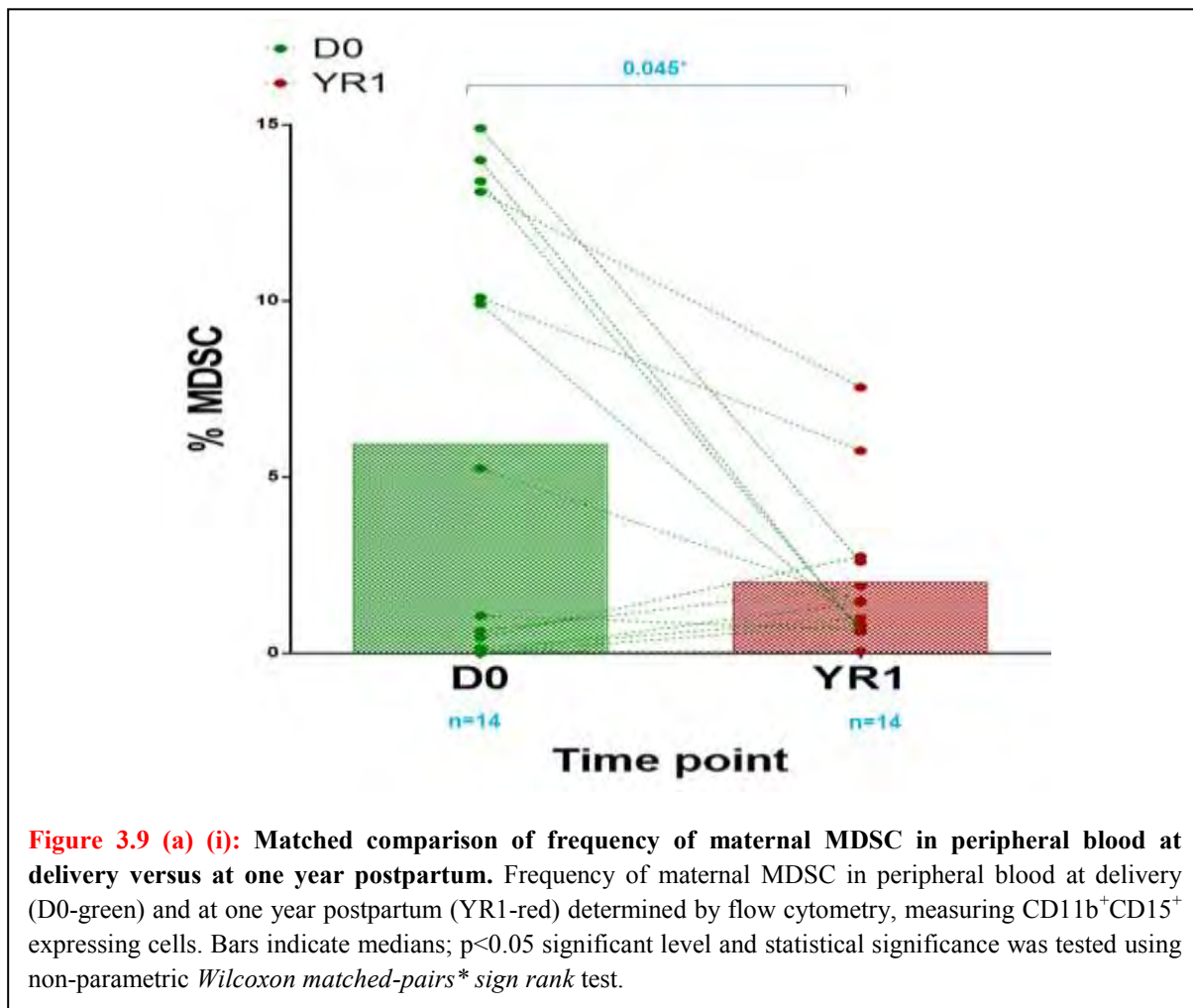


3.3.2 Quantification of maternal MDSC at delivery and one year postpartum

We evaluated the quantity of MDSC in mothers at time of delivery and at one year postpartum as described in [section 3.2](#).

3.3.2.1 Quantity and ontogeny of maternal MDSC at delivery and one year postpartum

In the matched time point analysis maternal MDSC frequency was significantly higher at delivery compared to one year postpartum (median MDSC D0=3.160% versus YR1=1.213% and $p=0.045$) [Figure 3.9 (a) (i)]. For this thesis we included a total of 70 mothers and their infants at delivery, however blood samples were only successfully obtained from 69 mothers at delivery and only 14 mothers and their infants had completed the study after one year follow-up postpartum from whom whole blood samples were collected [Figure 3.9 (a) (ii)].



Differences were not evident in maternal MDSC frequency between delivery and one year postpartum in the unmatched time point analysis including all 69 mothers enrolled at delivery and the 14 mothers at one year postpartum (median MDSC D0=1.010% versus YR1=1.465% and $p=0.342$) [Figure 3.9 (a) (ii)].

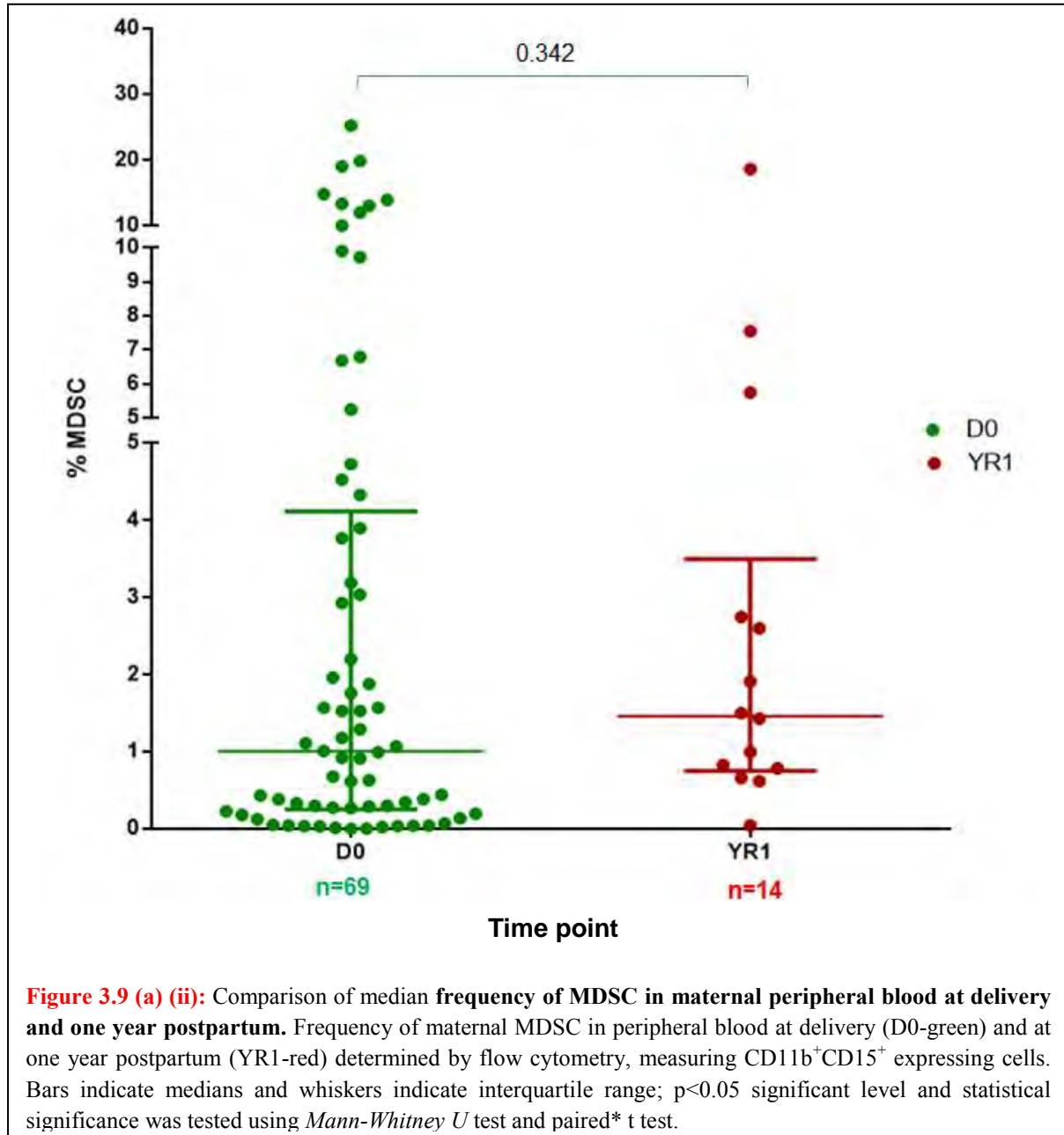
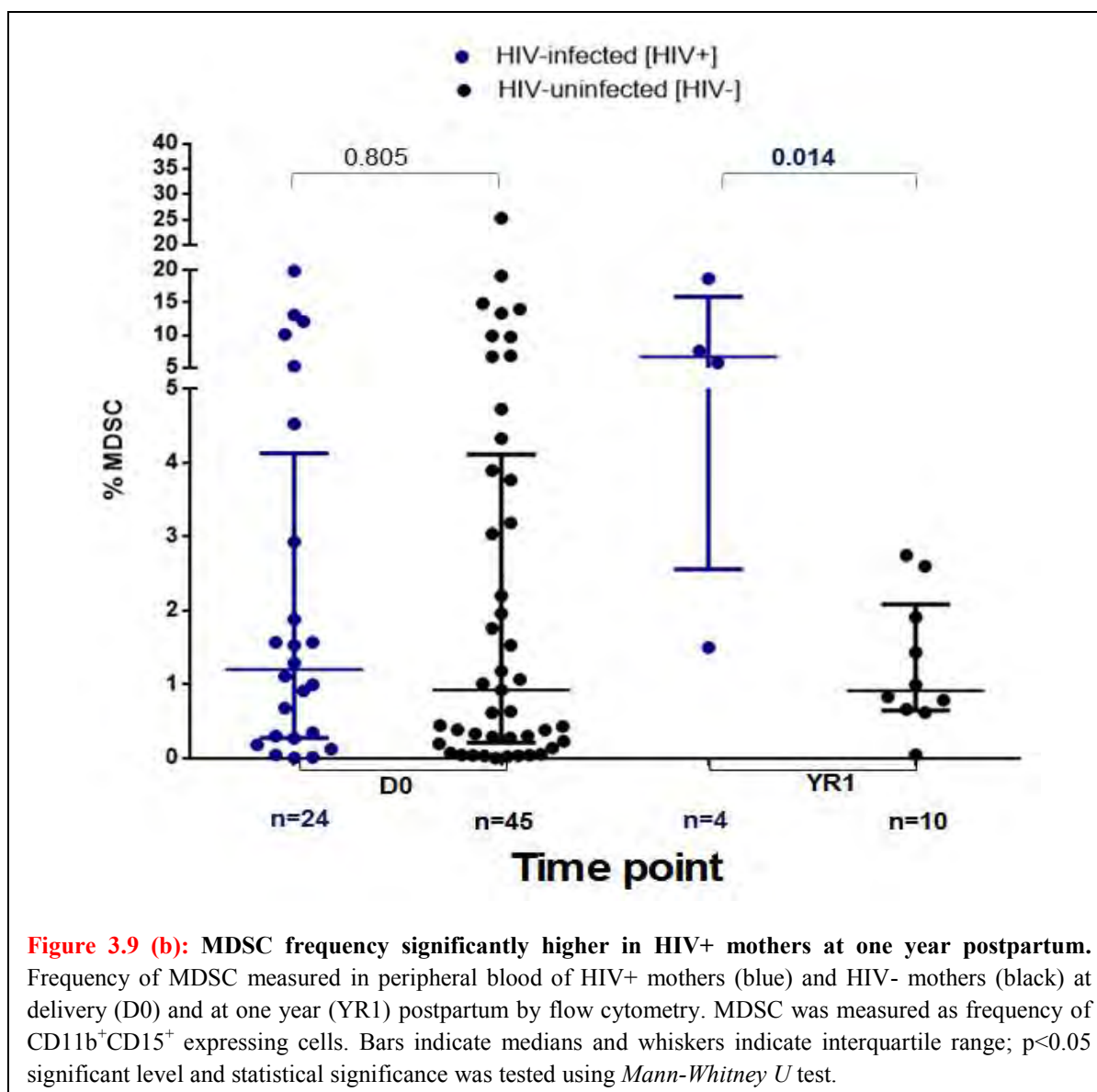


Figure 3.9 (a) (ii): Comparison of median frequency of MDSC in maternal peripheral blood at delivery and one year postpartum. Frequency of maternal MDSC in peripheral blood at delivery (D0-green) and at one year postpartum (YR1-red) determined by flow cytometry, measuring CD11b⁺CD15⁺ expressing cells. Bars indicate medians and whiskers indicate interquartile range; $p<0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test and paired* t test.

3.3.2.2 Presence and ontogeny of maternal MDSC in HIV+ compared to HIV- mothers

There was a significantly higher MDSC frequency in HIV+ mothers compared to their uninfected counterparts at one year postpartum (median MDSC HIV+ YR1=6.650% versus HIV- YR1=0.914% and $p=0.014$), but not at delivery (median MDSC HIV+ D0 =1.20% versus HIV- D0=0.923% and $p=0.805$) [Figure 3.9 (b)]. However, the number of HIV+ women that had completed 1 year of follow-up was small.



3.4 Discussion

Understanding cells that are involved in immune response regulation is critical for better vaccine development. The traditional T-regulatory (Tregs) cells have been well studied, and their influence on immune response regulation is well characterised in foetuses, neonates and infants (Gervassi *et al.*, 2014) (Mold *et al.*, 2008) (Wayne *et al.*, 2005) (Takahata *et al.*, 2004) (Mackroth *et al.*, 2004) (Mackroth *et al.*, 2004). There are potential ways of eliciting strong immune responses in neonates amidst the predominant regulatory mechanisms; these include understanding neonatal immunological characteristics such as the impact of Tregs and other suppressor cell populations, importantly Myeloid Derived Suppressor Cells (MDSC) on vaccine immunity that may contribute to the development of effective vaccination strategies. The persistence of these cells postnatally may have a negative effect on immunity and protection against infections.

To the best of our knowledge, there is no published study evaluating the quantity and ontogeny of MDSC in infants over time from birth to one year of age. In line with our hypothesis, over time, from birth through one year we found a significant decrease in MDSC frequency in infants [Figure 3.6 (b)]. Also, there were some distinct spikes in MDSC frequency for some infants particularly at 6 weeks and 6 months' time points when compared to preceding time points. The possible reasons may be that since infants are administered a bunch of vaccines at 6, 10 and 14 weeks of age, these vaccines may be involved in MDSC induction. Furthermore, these spikes in MDSC frequency may be non-specifically induced.

No study has compared MDSC frequency and ontogeny between HEU and HU infants. We found that HIV-exposure had little effect on MDSC frequency. However, there were few samples in the HEU infants group and this would be one of the limitations in the study. We had hypothesised that HEU infants are born from a highly activated immune environment and the increased frequency of regulatory immune cells during gestation (Rich *et al.*, 1997) (Kidzeru *et al.*, 2014) (Nair *et al.*, 2014), this effect may persist postnatally in HEU infants. However, we could not confirm our hypothesis. This may be because although born to HIV-infected mothers, the infants did not encounter any HIV antigen *in-utero* due to maternal antiretroviral therapy.

Also, it may be due to the limited number of HEU infants and a higher sample size would provide a better understanding.

Consistent with our hypothesis, maternal MDSC frequency was significantly higher at delivery compared to one year postpartum in the matched time point analysis [Figure 3.7 (a)]. This suggests that MDSC may be induced during pregnancy to maintain tolerance, and that they wane postpartum. In support of our findings, we and others have previously shown that the frequency of MDSC in mothers and foetuses is increased during gestation to allow tolerance between the mother and the foetus(Rieber *et al.*, 2013)(Gervassi *et al.*, 2014)(Nair *et al.*, 2014), and MDSC persist postnatally in infants(Gervassi *et al.*, 2014).

We found no significant difference in MDSC frequency between HIV+ versus HIV- mothers at delivery [Figure 3.7 (b)]. This could be due to the dominant influence of pregnancy-induced levels of MDSC masking the effect of HIV infection. In accordance with our hypothesis, at one year postpartum, HIV+ mothers had significantly higher MDSC frequency compared to their uninfected counterparts [Figure 3.7 (b)]. However, due to the small number of HIV+ mothers at one year follow-up time point, we recommend future experiments and a higher sample size to confirm this finding. In support of our findings, Vollbrecht *et al.* have shown that elevated levels of MDSC are associated with chronic uncontrolled HIV-infections(Vollbrecht *et al.*, 2012). Garg *et al.* also found an expansion of MDSC induced by the HIV-1 envelope protein gp120(Garg *et al.*, 2013).

We do not know what would happen at a further time point, say two years postpartum. The presence of high MDSC frequencies may be cleared over time. Since the study was performed in a high tuberculosis (TB) endemic area, maternal exposure to TB and helminths infections may be responsible for the MDSC levels in healthy African women(du Plessis *et al.*, 2013)(van Ginderachter *et al.*, 2010). Also, the women may have become pregnant again by one year post previous delivery and as one of the limitations of this study we did not have male controls to evaluate what is normal with this population of cells.

This study differs from other studies of MDSC in humans, particularly in the cohort characteristics. The cohort in Rieber *et al.* was different from ours and composed of cord blood

from healthy term neonates, infants at 2-16 years of age, both male and female healthy individuals(Rieber *et al.*, 2013). This was irrespective of whether the females donors recruited in the cohort were pregnant or not. Our cohort was made of women at delivery and they were followed up at one year postpartum. Our study and that of Reiber *et al.* was similar in that we evaluated the frequency of granulocytic MDSC (G-MDSC) cell population characterised as $CD11b^+cd14^-CD33^+CD15^+HLA-DR^-$ expressing cells(Rieber *et al.*, 2013). There were some differences between this study and that of Gervassi *et al.* The infants in this study were recruited from normal vaginal delivery exposed to maternal vaginal microbiome and some exposed to *in-utero* HIV-infection which may play a role in MDSC frequency, while the infants in the Gervassi *et al.* study were born from caesarean-sections and non-exposed to *in-utero* HIV-infection(Gervassi *et al.*, 2014).

Our data shows that the relative immunodeficiency in infants in the first few weeks of life could be secondary to MDSC, but that this effect should wane by one year of age. We showed a significant positive correlation between infants and maternal MDSC frequencies as well as absolute counts. This shows that, increased MDSC frequencies during gestation may persist postnatally in infants and decrease over time postnatally in both infants and mothers. Since this study was the first to show MDSC frequency over time in children, we recommend further studies addressing MDSC ontogeny to be performed for better understanding and interpretation. This would provide information that would contribute to guide the design of vaccine strategies in infants.

The study had a few limitations; the number of HEU infants as well as the HIV+ mothers that completed the study was small compared to the control group however, our primary interest was the general ontogeny of these cells. Furthermore, due to censoring of the cohort and high loss to follow-up, few infants and their mothers were included at the later time points.

The clinical importance of these findings remains unknown, but these data may have important implications as far as evaluating vaccine immune responses in infants. The high MDSC at birth may alter responses to vaccines administered at birth and should be taken into consideration when designing neonatal vaccines.

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CHAPTER 4

T CELL PROLIFERATIVE RESPONSES TO VACCINES AND ANTIGENS IN WHOLE PBMC AND WITHOUT MYELOID DERIVED SUPPRESSOR CELLS

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4.1 Introduction

It has been shown that infants have compromised vaccine immune responses in the first few weeks of life compared to older children and healthy adults (Gervassi *et al.*, 2014). Gasparoni *et al.* have shown that there is a gradual reduction of immunity and cellular mediated immune system immaturity during gestation, but a gradual development at birth in infants (Gasparoni *et al.*, 2003). There is evidence that T cell immuno-proliferation to mitogen concanavalin A (con A) is significantly lower in cord blood than in children and adults (Gasparoni *et al.*, 2003).

Several studies have shown evidence of immune suppression by MDSC (Poschke *et al.*, 2012) (Gabrilovich *et al.*, 2012) (Ostrand-rosenberg *et al.*, 2012) (Pillay *et al.*, 2013) (Serafini, 2013). The immune suppressive effect of MDSC on T cell proliferation to different antigens has been widely described in non-human primate (Green *et al.*, 2013) (Sui *et al.*, 2014) (Raber *et al.*, 2014) and cancer patients (Lindau *et al.*, 2012) (Gabrilovich *et al.*, 2012) (Raychaudhuri *et al.*, 2011).

There is limited data on the effect of MDSC on vaccine immune responses in infants. Most of the studies have looked at T cell proliferation in response to non-specific stimulation. We recently showed that, MDSC suppress *in vitro* T cell proliferation in neonates in response to anti-CD3CD28 (Dynabead) (Gervassi *et al.*, 2014). Also, Rieber and colleagues showed that after stimulation with a combination of IL-2 and muromonab-CD3 (OKT3), neonatal MDSC suppress adult T cell proliferation (Rieber *et al.*, 2013). Several studies have looked at the effect of MDSC on cellular immune responses mostly in adults with disease (Vollbrecht *et al.*, 2012) (Zeng *et al.*, 2014) (du Plessis *et al.*, 2013).

We and others have shown that the immune system of HIV exposed infants is compromised and makes them susceptible to infections (Epalza *et al.*, 2011), and alters their immune responses to vaccines (Miles *et al.*, 2010) (Jones *et al.*, 2011) (Slogrove *et al.*, 2012) (van Rie *et al.*, 2006) (Mazzola *et al.*, 2011) (Kidzeru *et al.*, 2014). We and others have also shown altered T cell proliferation in HEU infants; we showed a significant increase in T cell proliferation in HEU infants to BCG and non-specific stimulation with SEB compared to HIV-unexposed infants, but T cell proliferation to *Bordetella pertussis* stimulation was comparable between

groups(Kidzeru *et al.*, 2014). However, this increase in T cell proliferation observed was less protective as there was a significant reduction in cytokine production in HEU infants by proliferating T cell(Kidzeru *et al.*, 2014) And Mazzola *et al.* showed that HIV-exposure impairs BCG-specific T cell proliferation and cytokine responses in uninfected infants(Mazzola *et al.*, 2011).

HIV infection has been associated with immune activation much more than other disease states(Paiardini *et al.*, 2013)(Chang *et al.*, 2010)(Catalfamo *et al.*, 2011)(d'Ettorre *et al.*, 2011). In HIV-infected individuals, vigorous HIV-1 specific CD4⁺ T cell proliferation to viral protein p-24 have been observed(Rosenberg *et al.*, 2012). There is depletion in CD4⁺ T cells in chronic HIV infected patients due to immune activation(Hammond *et al.*, 2008)(Okoye *et al.*, 2013). HIV-1 antigen gp120 has been shown to induce expansion of MDSC in HIV infected individuals(Garg *et al.*, 2013). There is evidence that MDSC suppress T cell proliferation in HIV-infected individuals, reflecting an anti-proliferative response against the extreme immune activation during HIV infection(Qin *et al.*, 2013)(Gama *et al.*, 2012)(Nagarajet *al.*, 2013)(Vollbrecht *et al.*, 2012).

It was recently shown that MDSC reduced protective immune responses to SIV in SIV vaccinated macaques in a non-human primate macaque model(Sui *et al.*, 2014). However, little is known on how MDSC affect T cell proliferation in response to vaccines in humans generally. More specifically, there is little about the effect of MDSC in vaccine induced T cell proliferation in HIV-exposed uninfected (HEU) infants or in HIV+ individuals, and in mothers postpartum.

We compare T cell proliferative responses to vaccines using Tuberculin purified protein derivative (PPD), Tetanus toxoid (TT) and Hepatitis B surface antigen (HepB); cells alone with medium as negative control (CA), and stimulation with Dynabeads, a human T cell activator anti-CD3/CD28 as positive control (Dynabead, Life Technologies). We assess the consequences of MDSC on the development of antigen specific cellular proliferative immune responses between birth and one year in infants and in mothers at delivery and at one year postpartum. This was done by measuring the frequency of T cells with low expression of Carboxyfluorescein

Succinimidyl Ester (CFSE) stain i.e. CFSE_{low}-expressing CD4⁺ T-cells in whole cord blood mononuclear cells (CBMC) and/or peripheral blood mononuclear cells (PBMC), and MDSC depleted (CD15⁻) CBMC and/or PBMC in cultures after stimulating with optimized concentrations of the respective antigens. Hence, in this chapter we were interested in determining the potential effect of MDSC on T cell proliferation in response to vaccine antigens in infants and mothers from the time of delivery to one year postpartum.

4.2 Materials and method

Using the standard ficoll gradients, CBMC and PBMC were isolated as described above (Section 3.2.1). Where required, in some samples red blood cells (RBC) were depleted using glycophorin A micro beads (Section 3.2.2). Further, depending on the number of cells recovered, cells were divided into two aliquots; those for non-depletion (CD15⁺ ND) and MDSC depletion (CD15⁻). Depletion of MDSC was performed by depleting CD15⁺ cells from whole CBMC and/or PBMC by employing the EasySep[®] magnetic cell separation technique as described below (section 4.2.1).

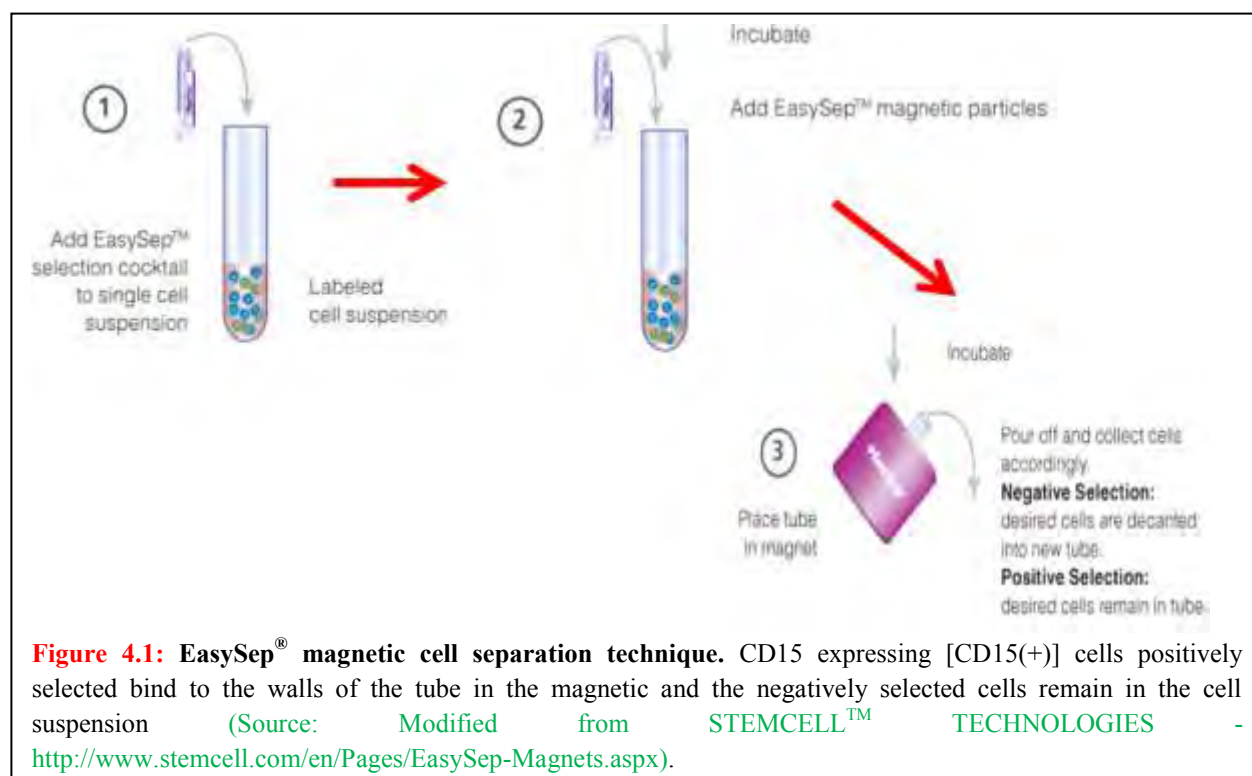
We prioritised experiments according to blood volume obtained, and often the volume of whole blood collected from infants was insufficient for CD15⁺ depletion, and the number of CBMC/PBMC not enough. Due to this, we prioritized experiments for which samples were provided since there were only a limited number of infants and their mothers at most time points for which depletion was able to be performed and further permitting MDSC non-depleted versus MDSC depleted match paired analyses to be performed. Table 4.1 describes how experiments were prioritized based on the total CBMC/PBMC isolated with respect to the volume of blood collected.

Table 4.1: Description of how experiments were prioritized based on the volume of blood and total CBMC/PBMC isolated. The three experiments performed (MDSC staining for characterisation of MDSC (Chapter 3 above), and CFSE proliferation assay (Chapter 4) with IFN- γ ELISpot assay (Chapter 5) both involving comparisons between CD15⁺ non-depleted versus CD15⁺ depletion (Depl.) experiment. Antigens of importance included Hep B, BCG and PHA.

Total CBMC/PBMC isolated & amounts for experiments prioritized based on importance and cells available (million cells)	1	2	3	4	5	6	7	8	9	10	11
MDSC stain	√	√	√	√	√	√	√	√	√	√	√
IFN- γ ELISpot assay		√	√		√	√	√	√	√	√	√
IFN- γ ELISpot with CD15 Depl.			√			√	√		√	√	√
CFSE (neg, Hep B, PHA)				√	√	√	√	√	√	√	√
CFSE with CD15 Depl. (neg, Hep B, PHA)								√	√	√	√
CFSE (BCG)										√	√
CFSE with CD15 Depl. (BCG)											√

4.2.1 EasySep[®] magnetic cell separation techniques

The EasySep[®] magnetic cell separation technique was performed as follows; in a 5mL FACS tube, cells were suspended in ice cold MACS separation buffer [0.1% heat inactivated human serum (HS) and 0.4% of 2mM EDTA, into 500mL phosphate buffered saline (PBS)] i.e. 1mL MACS buffer per 1×10^8 cells, plus 100uL EasySep[®] Custom Anti-Human CD15 TAC cocktail. The cell suspension was further incubated in the dark for 10 minutes. After, 100uL of EasySep[®] Magnetic Nanoparticles Positive Selection reagent was added to the mixture, and further

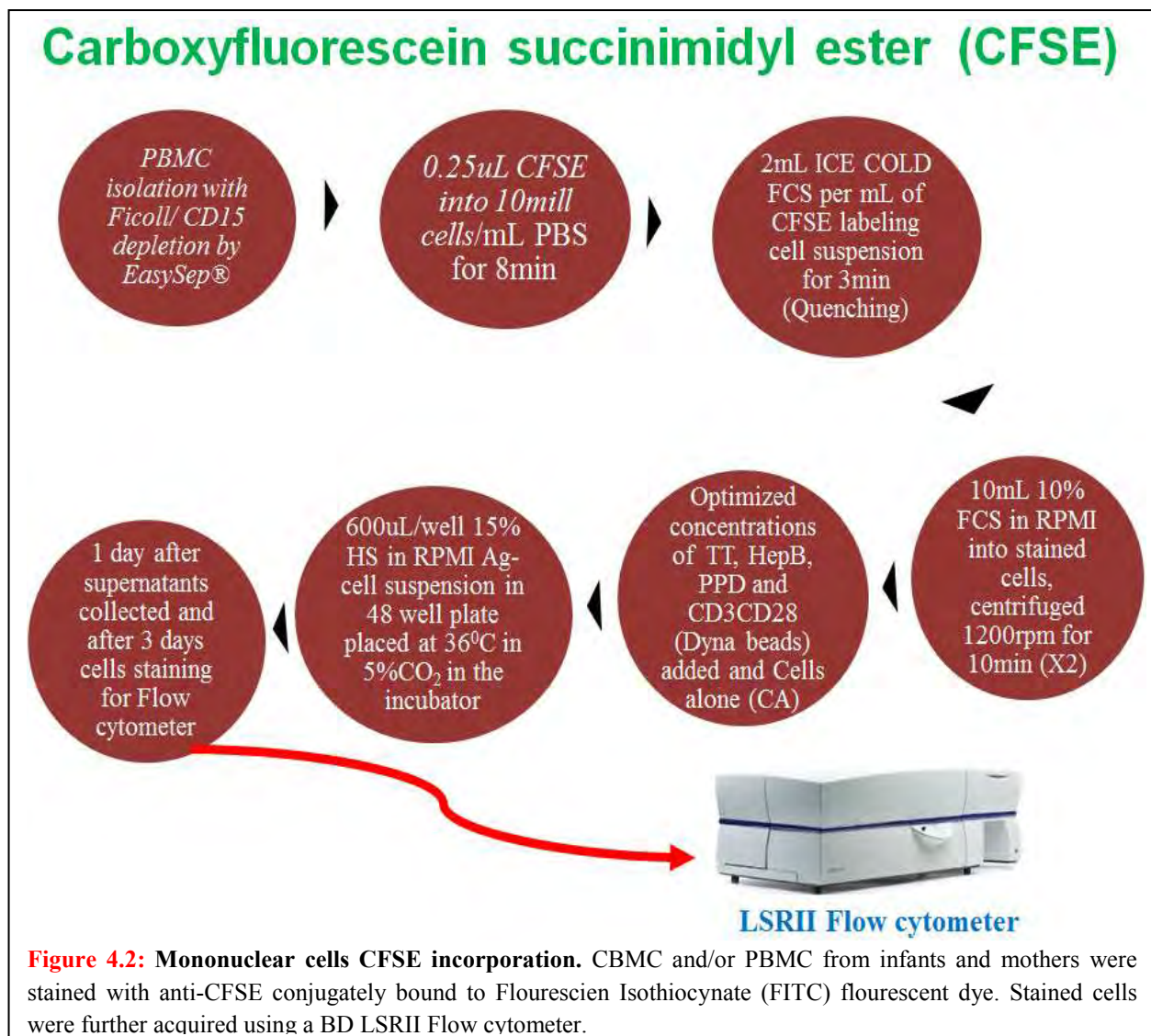


incubated for another 10 minutes. The suspension was then topped-up to 2.5mL with MACS separation buffer and the tube inserted in the EasySep[®] magnet for 10 minutes, without cap. Positively selected CD15⁺ cells bound to the walls of the tube, and CD15⁻ cells located in the cell suspension. After incubation, the negatively selected cells were decanted out of the tube with the tube still in the magnet (Figure 4.1). The depletion process was repeated twice, to complete three rounds of CD15 depletion.

4.2.2 Mononuclear cell CFSE incorporation technique

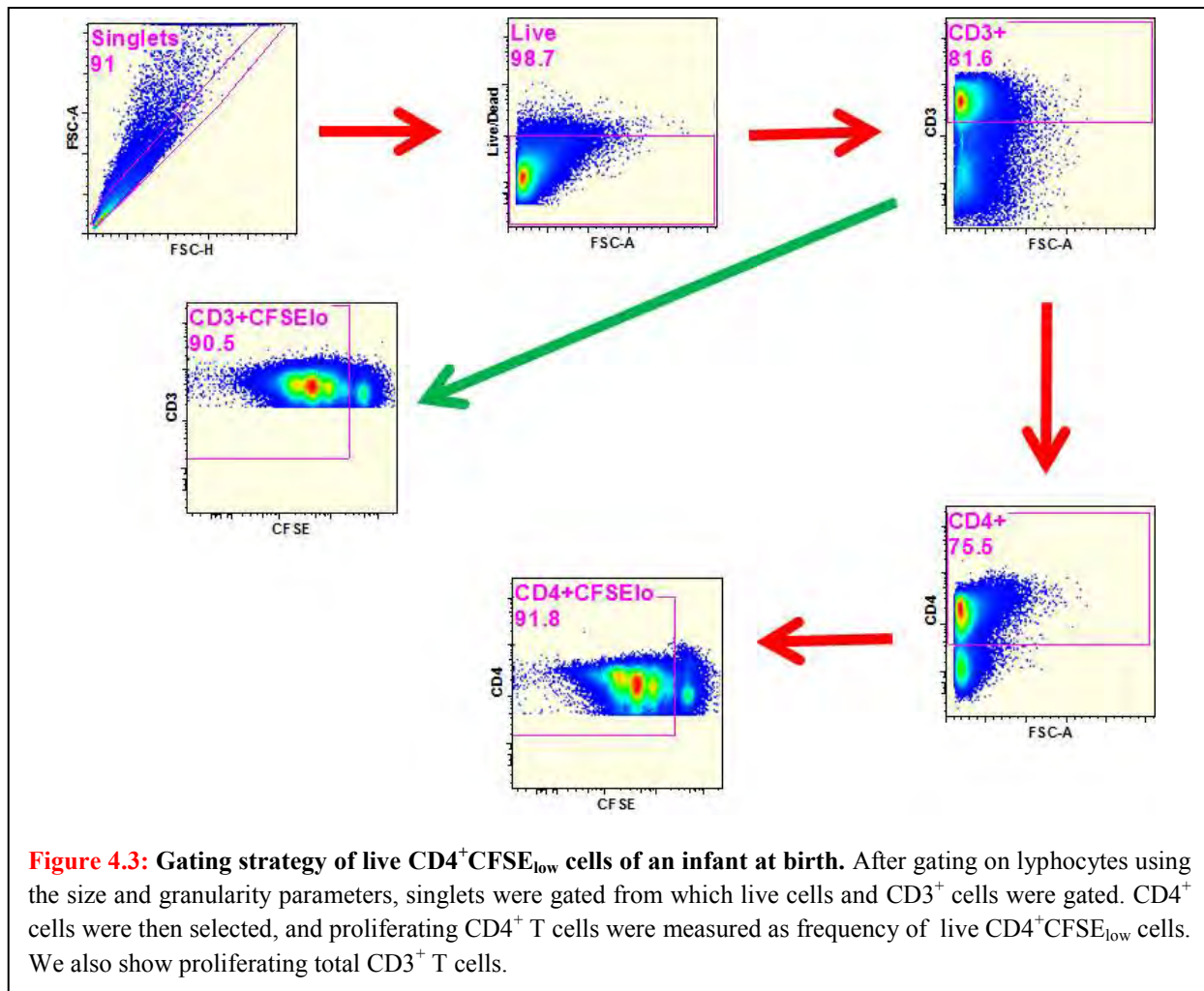
After obtaining all the populations of cells required for CFSE incorporation i.e. whole PBMC - CD15 non-depleted cells (CD15⁺), and absence of MDSC - CD15 depleted cells (CD15⁻); CFSE incorporation was performed.

Briefly, 0.25uL of Cell Trace™ CFSE (Life Technologies, USA) was added into 1×10^7 cells in 1mL PBS and incubated for 8 minutes in the dark at room temperature (RT). Thereafter, 2mL ice cold, heat inactivated foetal calf serum (FCS) was added to cells for an additional 3 minutes, to “quench” the CFSE, as CFSE has been shown to be highly toxic to cells(Quah *et al.*,



2007)(Wang *et al.*, 2005). After, 10mL of 10% FCS in Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma Aldrich) containing 1% penicillin-streptomycin antibiotics, was added into the stained cells to wash off the excess CFSE stain, and centrifuged at 1200 rpm for 10 minutes. The washing step was repeated once and the cell pellet re-suspended (Figure 4.2).

Using a 48 well flat bottom plate, 1×10^6 cells/well of the different cell populations i.e. whole CBMC and/or PBMC with and without MDSC, were plated, and incubated for 2-3 days in 600uL/well RPMI culture medium containing antigen; 1% penicillin-streptomycin, 15% Human Serum and 0.02% of 0.5ug/mL recombinant human IL-7 (rhIL-7), in a 37°C, 5% CO₂ humidified incubator. Daily, 0.1% of 0.5ug/mL IL-7 was added per well for the rest of the incubation period as it is required to enhance the survival and development of cells(Rathmell *et al.*, 2001)(Cieri *et*



al., 2013)(Sara *et al.*, 2002)(van Lent *et al.*, 2009). Optimized concentrations of the respective antigens were used, in order to measure the frequency of proliferating CFSE_{low}-expressing CD4⁺ T-cells (i.e. CD4⁺CFSE_{low}) in response to various vaccines and antigens in infants and their mothers.

Specifically, the following antigens were added: 1ug/mL Tuberculin purified protein derivative (PPD) [STATENS SERUM INSTITUT, DK-2300, Copenhagen, Denmark], 0.25ug/mL Tetanus toxoid (TT) [TETAVAX, Aventis Pharma (Pty) Ltd], and 5ug/mL Hepatitis B surface antigen (HepB) [Advanced Immunochemical Inc.]. Cells alone without any stimuli served as a negative control (CA), and 5ug/mL Dynabeads[®], a human T cell activator CD3/CD28 served as positive control [Life Technologies]. In the wells with TT and HepB antigens, 0.01ug/mL and 0.5ug/mL BD Fastimmune[™] CD28/CD49d co-stimulation was added respectively.

T cell proliferation with and without MDSC was compared. Using a 96 well round bottom plate, antigen specific CFSE_{low} proliferating T cells were stained.

After 2-3 days cell culture, the cells were transferred to a pre-labelled 96 well round bottom plate. Extracellular staining with an optimized concentration of 50uL Violet fluorescent reactive dye (VIVID) [Life Technologies] was performed; cells were incubated for 10 minutes in the dark at RT, to stain dead cells. After incubation, the cells were washed off the excess VIVID with 200uL of 2% FACS wash buffer i.e. 2% Human Serum in PBS, and the plate was centrifuged at

2100rpm, repeated once. Cells were further fixed with 100uL of 10% BD FACS[™] Lysing Solution, in distilled H₂O, and incubated for 10 minutes. After

Table 4.2: The CFSE multicolour flow cytometry panel. The four colour flow cytometry panel employed with the various antibodies markers, fluorochromes, their suppliers and rationale (importance for inclusion in the panel).

Antibody	Fluorophore	Clone	Supplier	Rationale
VIVID	Pacific Blue	-	Life technologies	Dead cells exclusion
CD3	APC-Cy7	SK7	BD Biosciences	T cells
CD4	APC	L200	BD Biosciences	T cells
CFSE	FITC	-	Life technologies	Proliferating cells

incubation, the cells were washed with 200uL of 2% FACS wash buffer, repeated once. Further, using 200uL of 10% BD FACS[™] Permeabilizing Solution II, in distilled H₂O; cells were

permeabilized, and incubated for another 10 minutes. After incubation, the plate was centrifuged, and the cells washed with 200uL of 2% FACS wash buffer, repeated once. The cells in the plate were then stained with optimized concentrations of the respective fluorochromes bound antibodies, to stain proliferating T cells ($CD4^+CFSE_{low}$) (Table 4.2). Responses were measured using BD LSRII flow cytometer and analysis with FlowJo v9.4.7.

Based on the amount of whole blood sample obtained, the number of CBMC/PBMC yield was prioritized per experiment if the volume was insufficient for all assays. Hence, the number of cells used for every assay was not the same; for CFSE, 4 million cells were required for the assay to be performed. Where the number of CBMC/PBMC was 4 million and above CFSE assay preferentially performed (where at least Hep B proliferative responses were prioritized to be assessed, including the cells alone and Dynabead wells as negative and positive controls respectively) to all the other assays that are less cell intensive i.e. a million cells required for MDSC staining and 2 – 3 million cells for IFN- γ ELISpot assay.

4.2.2.1 Data analysis

Flow cytometry data was extracted for analysis after sample acquisition. Below is a summary of the data analysis strategy.

After sample acquisition by BD LSRII, the FCS files were first analyzed using FlowJo v9.4.7 (Tree Star, College Park). After gating for singlets (using the size and granularity parameters) and live cells, we gated on $CD3^+$ cells, from which $CD4^+$ cells were gated. Finally, proliferating T cells were gated as $CFSE_{low}$ against $CD4^+$ cells (Figure 4.3). The negative and positive controls were employed to decide where gates were to be placed as T cell proliferative responses varied between antigens as well as between individuals. Final decision on placing the gates was made and the same gates applied to the other samples to keep consistency.

Since it is an exploratory study, investigating $CD4^+$ T cells is due to the fact that $CD4^+$ T cells have been shown to be rapidly activated post stimulation with the specific antigens and vaccines we assessed in this current study and proliferate at a higher rate than $CD8^+$ T cell (Caggiari *et al.*, 2001)(Tham *et al.*, 2002). This would provide a better understanding of the effect of MDSC on T cell proliferation to these vaccines antigens (BCG, PPD, BP, TT and HepB).

4.2.2.2 Standard rules for cut-offs and controls for the flow cytometry data

Following analysis by FlowJo, the data was exported and organized for further analysis. In order to prevent data bias and to perform background subtraction, the following rules were put in place:

1. Assay validity and positivity (before background subtraction)

- A. The test antigens (i.e. PPD, TT, HepB and Dynabeads) % $CD4^+CFSE_{low}$ were only quantified if the negative control (i.e. Media) % $CD4^+CFSE_{low}$ proliferating T cells were $\leq 5\%$, or the sample was excluded.

$$i.e. \% CD4^+CFSE_{low} [Media\ or\ cell\ alone] \leq 5$$

- B. The frequency of $CD4^+CFSE_{low}$ proliferating T cells in response to Dynabeads had to be greater than the median plus 3 times the MAD of the negative control, or the sample was excluded.

$$i.e. \% CD4^+CFSE_{low} [Dynabeads] > \% CD4^+CFSE_{low} (Median + 3MAD) [Media]$$

$$[MAD=Median\ Absolute\ Deviation]$$

MAD is defined as the deviation of each sample's negative control frequency from the negative control's median, the MAD is the median of the absolute values of the deviation (Rouss *et al.*, 1993).

2. Proportion of proliferating cells ($CD4^+CFSE_{low}$)

The frequency of $CD4^+$ T cells with low CFSE expression of the test antigens (PPD, TT, HepB and Dynabeads) minus background (% $CD4^+CFSE_{low}$ T cells in the media control)

$$i.e. (PPD, TT, HepB\ and\ Dynabeads\ induced\ \% CD4^+CFSE_{low}\ cells) - (\% CD4^+CFSE_{low}\ cells\ in\ the\ media)$$

3.) CD15-depletion ($CD15^-$) purity

Only samples with CD15-depletion purity of 90% and above were included in the data for further analysis (Appendix 4.3).

4.2.2.3 Statistical considerations

Different statistical packages were used to complete the data analysis after obtaining data from flow cytometry as listed in the table below (Table 4.3).

Table 4.3: Statistical packages used for data analysis

N°	SOFTWARE PROGRAM	ANALYSIS TYPE	DATA TYPE
1	FLOWJO v9.4.7 (for MAC, Stanford University, 1995-96; Tree Star, Inc. 1997-2012)	To analyze flow cytometry data after acquisition	Flow cytometry data analysis
2	Microsoft Excel 2013	To clean and organize data	
3	Microsoft Excel 2013	To perform background subtraction	
5	GraphPad Prism v5 (for Windows, GraphPad Software, San Diego California USA, www.graphpad.com”).	To analyze differences in T cell proliferation cells between groups.	Graphics -(CD15 ⁺ vs. CD15 ⁻) -(HEU vs. HU)
6	STATA v11 (for windows, StataCorp LP, College station, TX77845, USA).	Statistical tests for cohort description	-Chi-square test -Fisher's exact test -Wilcoxon rank sum test (Mann-Whitney test)

4.2.2.4 Statistical data analyses

Data analysis was performed by applying the following statistical tests;

Table 4.4: Statistical tests used to complete data analyses

Statistical test	Rationale	Statistical package	Data type
Chi-square test	Relationship between two categorical variables	STATA v11	Cohort description (HEU vs. HU)
Fisher's exact test	Relationship between two categorical variables (one or more of the values in the cells has an expected frequency ≤ 5)	STATA v11	Cohort description (HEU vs. HU)
Wilcoxon rank sum test (Mann-Whitney test)	Compare continuous data between two categories that is not normally distributed.	- GraphPad Prism v5 - STATA v11	-T cell Proliferation (CD15 ⁺ vs. CD15 ⁻) (HEU vs. HU)
Wilcoxon matched-pairs sign rank test	Compare continuous data between two matched categories that is not normally distributed.	- GraphPad Prism v5	-T cell Proliferation (CD15 ⁺ vs. CD15 ⁻)
Spearman Correlation	Correlations between two continuous variables	- GraphPad Prism v5	(MDSC frequencies vs. immune responses)

4.3 Results

In this chapter, we assessed the effect of MDSC on T cell proliferative responses to TT and HepB vaccination, to PPD (for BCG vaccination) and Dynabead antigens, in whole CBMC and/or PBMC compared with versus without MDSC, in HEU as well as HIV-unexposed (HU) infants, and in HIV-infected (HIV+) as well as HIV-uninfected (HIV-) mothers postpartum. T cell proliferative responses were assessed in infants at birth, 6, 10 and 14 weeks, 6 and 9 months, and at one year of age; and in mothers only after delivery and one year postpartum.

This chapter aimed to test the *hypothesis that in the presence of MDSC, infant and adult T cells will have low proliferative ability in response to vaccines in early life. Furthermore, we hypothesised to increased frequencies or higher suppressive abilities of MDSC, HEU infants and their HIV-infected (HIV+) mothers, will have lower proliferation in the presence of MDSC compared to the HIV-unexposed (HU) infants and HIV-uninfected (HIV-) mothers respectively.*

To better understand the effect of MDSC on T cell proliferation, we employed two analytical processes;

1. We performed binary analyses by comparing MDSC frequency of live PBMC as well as MDSC absolute counts per million PBMC between vaccine “responders” versus “non-responders” based on their CD4⁺ T cell proliferative response to vaccine antigens. The frequency of CD4⁺ T cell proliferation in response to vaccine antigens was considered as responder when proliferative responses were greater than or equal to 2.5%/10⁶ PBMC after back ground subtraction of the negative control responses from that of the test antigens. The basis of our consideration of 2.5%/10⁶ PBMC was due to the fact that CD4⁺ T cell proliferation was generally low in response to vaccine stimulations, and is supported by the finding by Soares *et al.* that the dye dilution of Oregon Green (OG), a CFSE derivative had an intra assay variability of 2-3% for CD4⁺ T cells (Soares *et al.*, 2010).

2. (a) We compared $CD4^+$ T cell proliferation to vaccines and antigens in PBMC (presence of MDSC) versus without MDSC (after MDSC depletion, only if depletion was considered successful ie. $\geq 90\%$) at all individual time points (i.e. at birth, at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age), and in mothers (i.e. at delivery and one year postpartum).
- (b) In infants, we evaluated the ontogeny of the effect of MDSC on $CD4^+$ T cell proliferation to vaccines and antigens in a grouped analysis.

We grouped close follow-up time points together to increase the sample sizes. For Hep B and TT, which is given first at 6 weeks of age, we grouped the 10 and 14 week time points (after the first and second vaccine doses and before the last vaccine doses), and the 6, 9 months and one year time points (after the last vaccine doses and the last follow-up time points). However, for PPD, since BCG is given at birth, follow-up ages were grouped into two, at 6, 10 and 14 weeks (first few weeks after priming and peak response(Kagina *et al.*, 2009)), and at 6 and 9 months and one year (the last follow-up time points).

Generally, vaccine responses to the different antigens varied. Also, responses varied between infants and their mothers. [Appendix 4.2](#) shows the general ontogeny of each vaccine response (median frequency of proliferating cells) to the different antigens in infants and mothers through time.

Hep B CD4⁺ T cell proliferative responses peaked at 10 weeks after priming at 6 weeks in infants and then dropped at 14 weeks post second vaccine dose. The responses then further rose after the last and third vaccine dose at 14 weeks then varied till one year of age [[Appendix 4.2, Figure G \(i\)](#)]. In the mothers, [Figure G \(ii\)](#), Hep B responses increased from delivery to one year postpartum.

TT CD4⁺ T cell proliferative responses were low throughout, from priming at 6 weeks of age, through one year of age [[Appendix 4.2, Figure H \(i\)](#)]. Likewise in mothers, [Figure H \(ii\)](#), TT responses were low from delivery to one year postpartum.

PPD responses peaked between 6, 10 and 14 weeks after BCG vaccination at birth in infants to 9 months and one year of age [[Appendix 4.2, Figure I \(i\)](#)], as previously described ([Kagina et al., 2009](#)). In mothers, [[Appendix 4.2, Figure I \(ii\)](#)], PPD responses increased from delivery to one year postpartum.

As expected of a positive control and strong stimulation, Dynabead induced T cell proliferation at all the time points were robust in both infants and mothers [[Appendix 4.2, Figure J](#)].

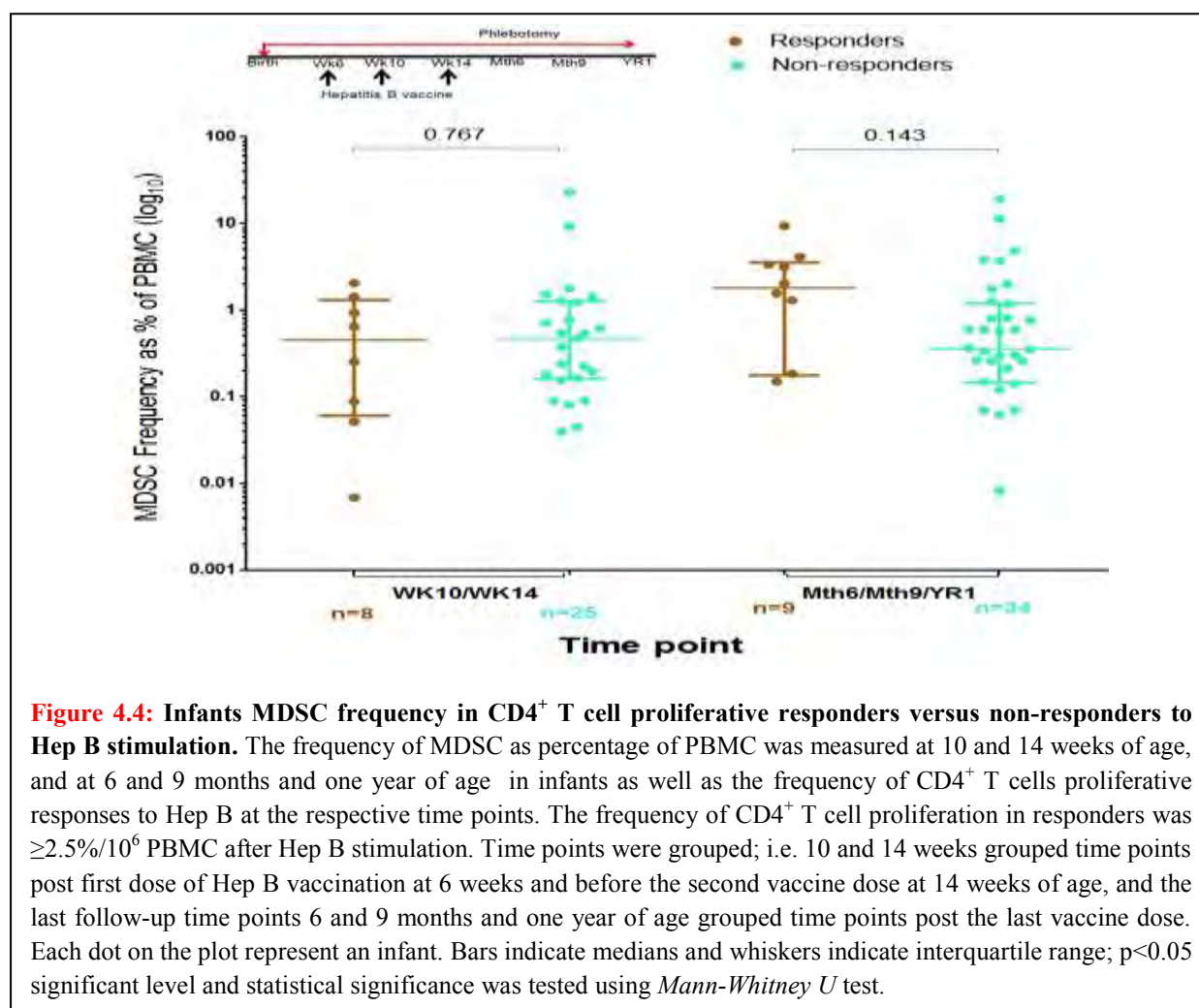
4.3.1 Infant MDSC frequency and absolute counts in responders versus non-responders as measured by CD4⁺ T cell proliferation

We evaluated the relationship between the frequency and absolute count of MDSC versus the frequency of CD4⁺ T cell proliferation in response to vaccines and antigens from birth to one year of age in infants.

To assess the degree at which MDSC may affect T cell proliferation, we evaluated the frequency and the absolute count of MDSC in infants that responded and that that did not respond as stated above in [Section 4.3 \(1\)](#). Also, we evaluated maternal MDSC frequency of CD4⁺ T cell proliferation to antigen stimulation in mothers who responded versus non-responders. [Table 4.5](#) below shows the summary of the results.

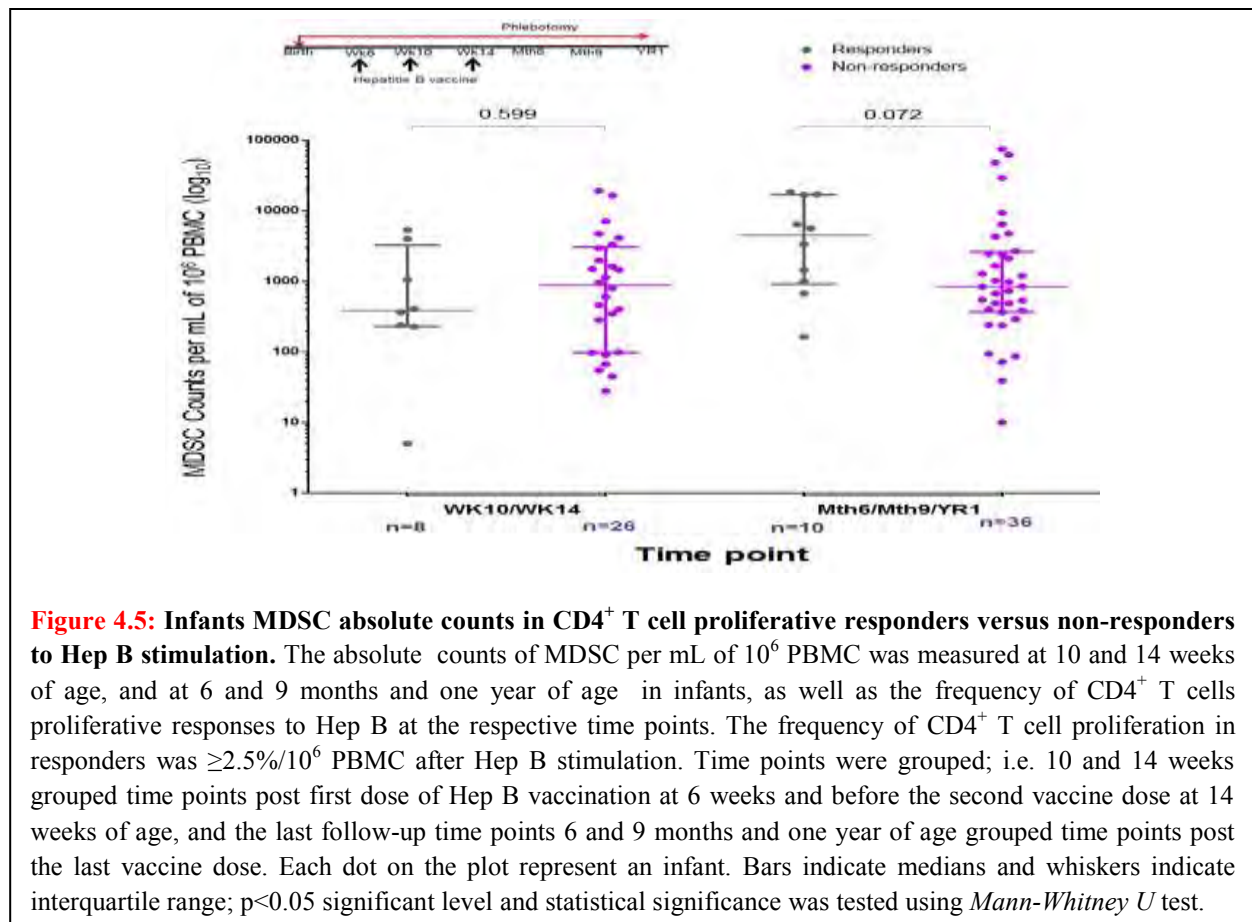
4.3.1.1 Infant MDSC frequency in responders versus non-responders to Hep B stimulation as measured by CD4⁺ T cell proliferation

The frequency of MDSC after Hep B vaccine stimulation in infants whose CD4 T cell proliferation was responsive versus non-responders was measured in the grouped time point analysis at 10 and 14 weeks grouped time points, and at 6 and 9 months and one year of age grouped time points, as there were too few data in the analysis at individual time points. As shown in Figure 4.4, there were no significant differences in MDSC frequency between responders and non-responders at all grouped time points (median of responders=0.451% versus non-responders=0.472% and $p=0.767$ at 10 and 14 weeks; median of responders=0.180% versus non-responders=0.359% and $p=0.143$ at 6 and 9 months and one year).



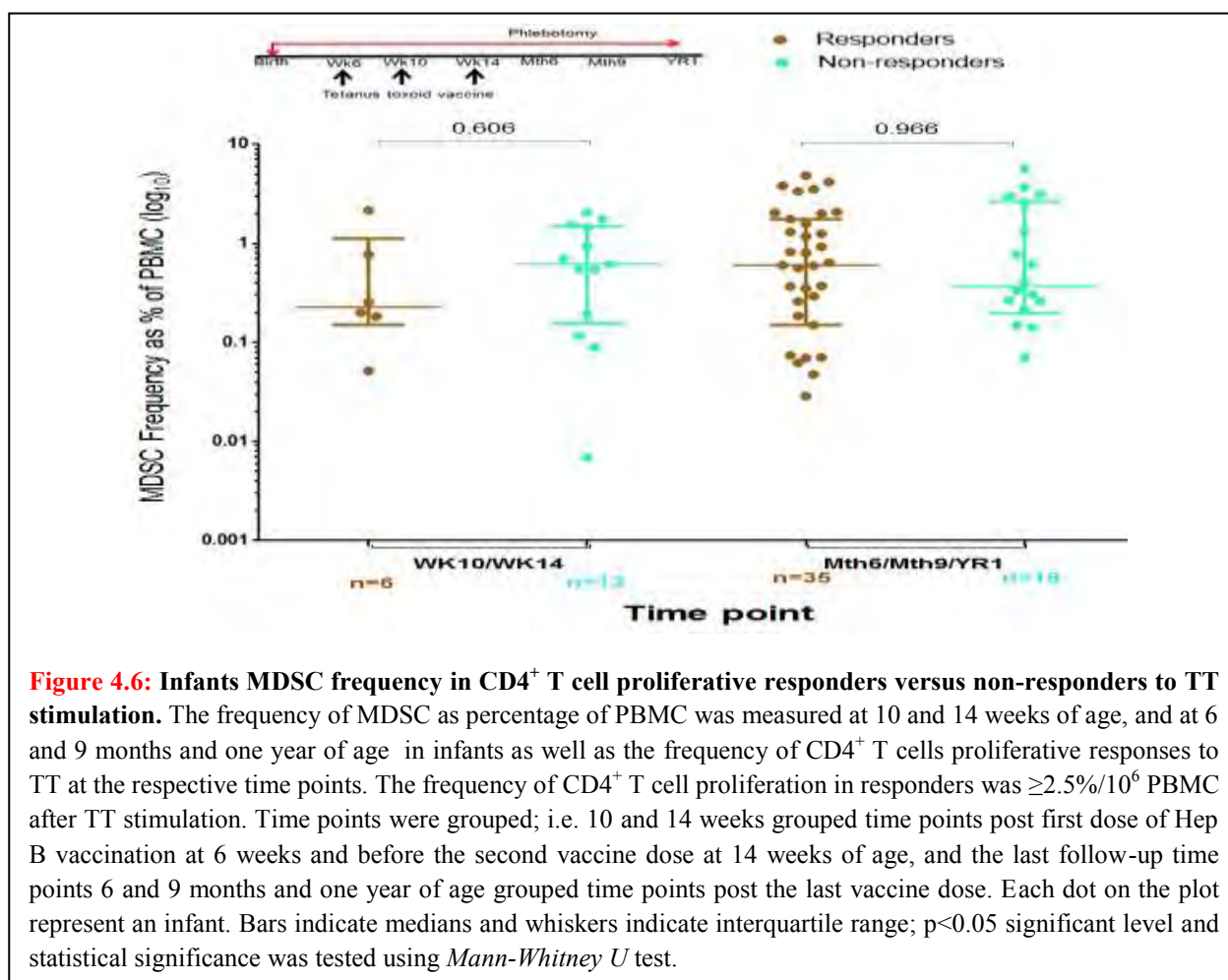
4.3.1.2 Infant MDSC absolute counts in responders versus non-responders to Hep B stimulation as measured by CD4⁺ T cell proliferation

The absolute MDSC counts after Hep B vaccine stimulation in infants who responded were compared to infants who responded poorly in terms of CD4⁺ T cell proliferative responses. 10 and 14 week time points were grouped, as well as 6 and 9 months and one year of age. There were no significant differences between responder and non-responder MDSC counts at all grouped time points; at 10 and 14 weeks (median of responders=386.0 MDSC counts versus non-responders=882.5 MDSC counts and $p=0.599$), and at 6 and 9 months and one year (median of responders=4511.0 MDSC counts versus non-responders=846.0 MDSC counts and $p=0.072$) (Figure 4.5). However, there was a trend towards high MDSC counts in the non-responders with CD4⁺ T cell proliferation $\geq 2.5\%$ at 6, 9 months and one year follow-up grouped time points post Hep B vaccination.



4.3.1.3 Infant MDSC frequency in responders versus non-responders to TT stimulation as measured by $CD4^+$ T cell proliferation

The frequency of MDSC in infants who responded versus non-responders in terms of $CD4^+$ T cell proliferative responses $\geq 2.5\%/10^6$ PBMC to TT were compared in the grouped time point analysis at 10 and 14 weeks grouped time points, and at 6 and 9 months and one year of age grouped time points. As shown in Figure 4.6, there were no significant differences between responders' and non-responders' MDSC frequencies at all grouped time points at 10 and 14 weeks (median of responders=0.227% versus non-responders=0.619% and $p=0.606$), and at 6 and 9 months and one year (median of responders=0.601% versus non-responders=0.369% and $p=0.966$).



4.3.1.4 Infants MDSC absolute counts in responders versus non-responders to TT stimulation as measured by CD4⁺ T cell proliferation

The absolute MDSC counts in infants who responded compared to infants who did not with regards to TT poorly was measured at 10 and 14 weeks grouped time points, and at 6 and 9 months and one year of age grouped time points. There were no significant differences between responders' and non-responders' MDSC counts at all grouped time points; at 10 and 14 weeks (median of responders=1649.0 MDSC counts versus non-responders=1476.0 MDSC counts and $p=0.325$), and at 6 and 9 months and one year (median of responders=3164.0 MDSC counts versus non-responders=1925.0 MDSC counts and $p=0.855$) (Figure 4.7).

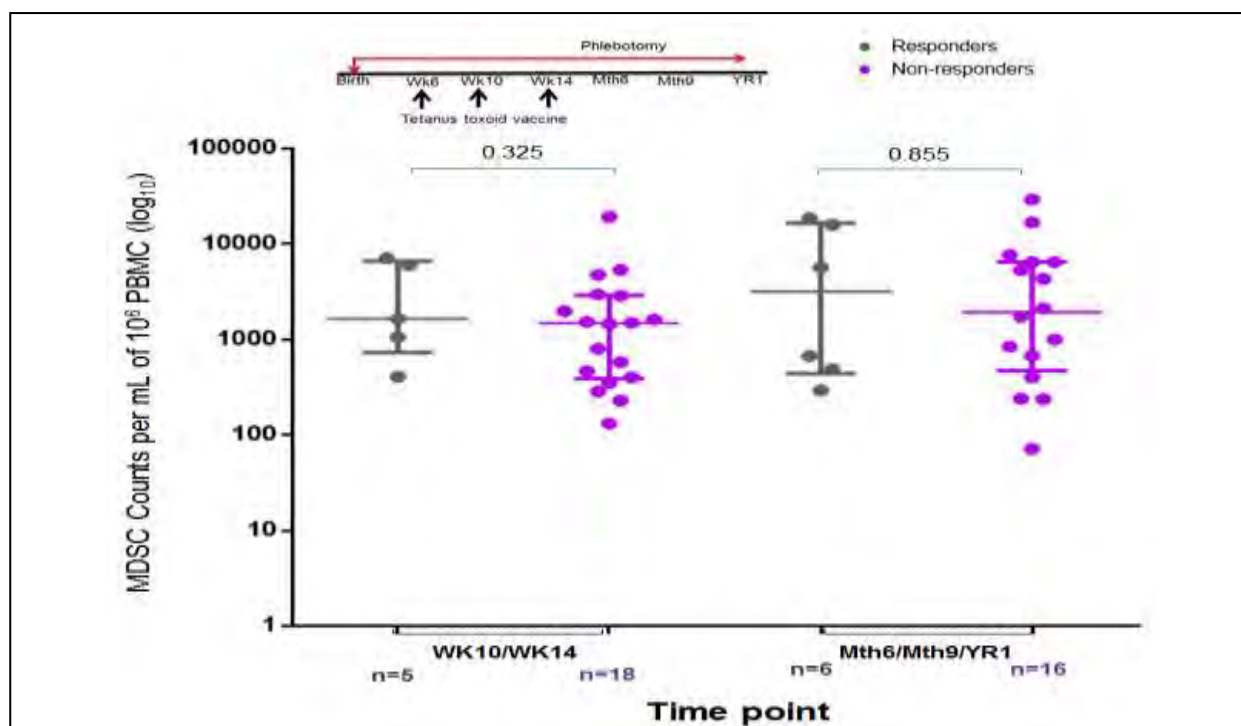


Figure 4.7: Infants MDSC absolute counts in CD4⁺ T cell proliferative responders versus non-responders to TT stimulation. The absolute counts of MDSC per mL of 10⁶ PBMC was measured at 10 and 14 weeks of age, and at 6 and 9 months and one year of age in infants, as well as the frequency of CD4⁺ T cells proliferative responses to TT at the respective time points. The frequency of CD4⁺ T cell proliferation in responders was $\geq 2.5\%/10^6$ PBMC after TT stimulation. Time points were grouped; i.e. 10 and 14 weeks grouped time points post first dose of TT vaccination at 6 weeks and before the second vaccine dose at 14 weeks of age, and the last follow-up time points 6 and 9 months and one year of age grouped time points post the last vaccine dose. Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

4.3.1.5 Infant MDSC frequency in responders versus non-responders to PPD stimulation as measured by CD4⁺ T cell proliferation

The frequency of MDSC in infants who responded and non-responders in terms of CD4⁺ T cell proliferative responses after PPD stimulation was compared in the grouped time point analysis at 6, 10 and 14 weeks, and at 6 and 9 months and one year of age. As shown in **Figure 4.8**, there were no significant differences between responders' and non-responders' MDSC frequencies at both grouped time points; 6, 10 and 14 weeks (median of responders=0.254% versus non-responders=0.169% and $p=0.246$), and at 6 and 9 months and one year (median of responders=0.589% versus non-responders=0.266% and $p=0.365$).

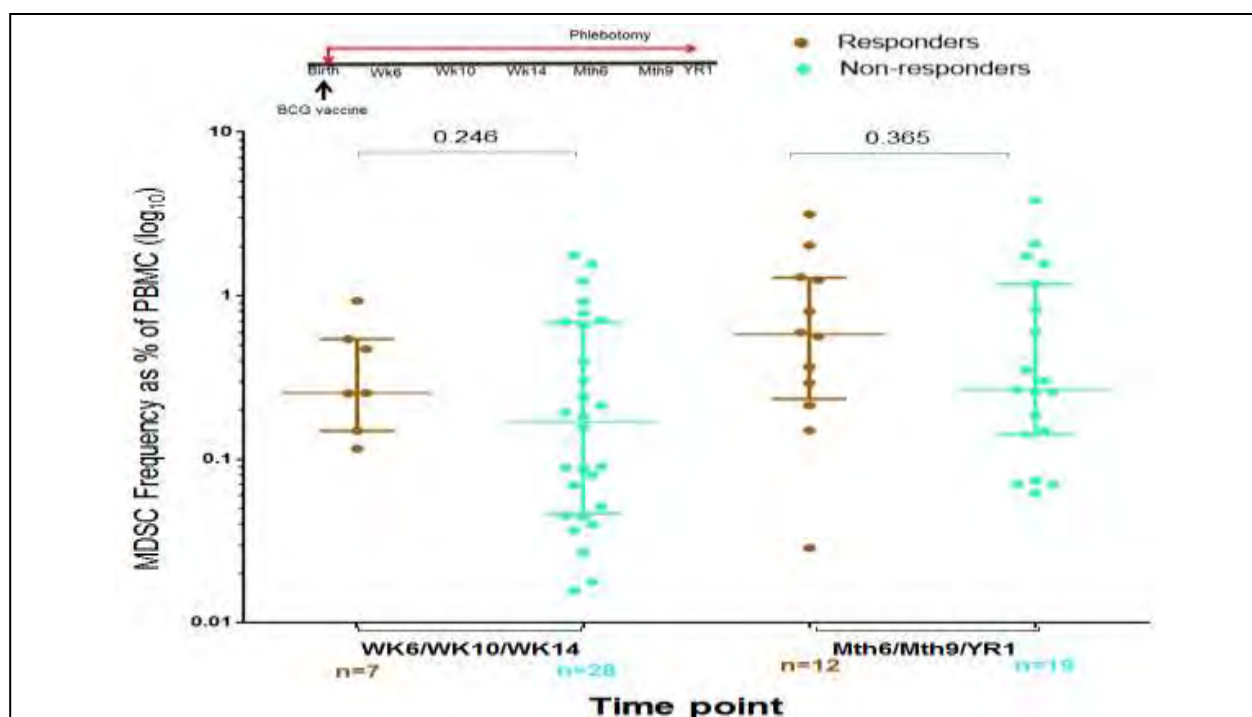


Figure 4.8: Infants MDSC frequency in CD4⁺ T cell proliferative responders versus non-responders to PPD stimulation after BCG vaccination. The frequency of MDSC as percentage of PBMC was measured at 6, 10 and 14 weeks of age, and at 6 and 9 months and one year of age in infants as well as the frequency of CD4⁺ T cells proliferative responses to PPD at the respective time points. The frequency of CD4⁺ T cell proliferation in responders was $\geq 2.5\%/10^6$ PBMC after PPD stimulation. Time points were grouped; i.e. 6, 10 and 14 weeks grouped time points post BCG vaccination at birth, and at the last follow-up time points 6 and 9 months and one year of age grouped time points. Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

4.3.1.6 Infants MDSC absolute counts in responders versus non-responders to PPD stimulation as measured by CD4⁺ T cell proliferation

The absolute counts of MDSC in infants who responded compared to infants who poorly responded in terms of CD4⁺ T cell proliferative responses to PPD stimulation was compared in the grouped time point analysis at 6, 10 and 14 weeks grouped time points, and at 6 and 9 months and one year of age grouped time points. There were no significant differences between responders' and non-responders' MDSC counts at 10 and 14 weeks grouped time points (median of responders=345.5 MDSC counts versus non-responders=250.0 MDSC counts and $p=0.726$), and at 6 and 9 months and one year (median of responders=1356.0 MDSC counts versus non-responders=673.0 MDSC counts and $p=0.884$) (Figure 4.9).

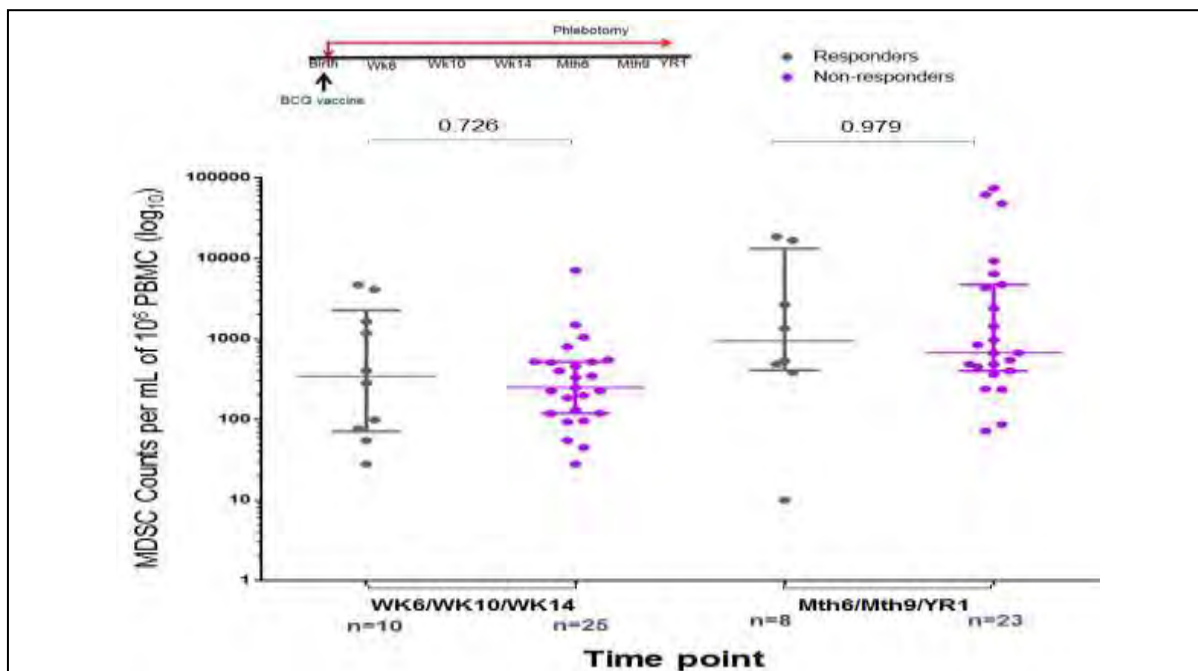


Figure 4.9: Infants MDSC absolute counts in CD4⁺ T cell proliferative responders versus non-responders to PPD stimulation after BCG vaccination. The absolute counts of MDSC per mL of 10^6 PBMC was measured at 6, 10 and 14 weeks of age, and at 6 and 9 months and one year of age in infants, as well as the frequency of CD4⁺ T cells proliferative responses to PPD at the respective time points. The frequency of CD4⁺ T cell proliferation in responders was $\geq 2.5\%/10^6$ PBMC after PPD stimulation. Time points were grouped; i.e. 6, 10 and 14 weeks grouped time points post BCG vaccination at birth, and at the last follow-up time points 6 and 9 months and one year of age grouped time points. Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

Overall, in infants MDSC frequency and absolute count between infants who were responders and non-responders to vaccine induced T cell proliferation were not significant at most time points. MDSC frequency and count varied between responders and non-responders to different vaccine antigens and at different grouped time points.

4.3.1.7 Maternal MDSC frequency in responders versus non-responders to Hep B stimulation as measured by CD4⁺ T cell proliferation

The frequency of maternal MDSC was not significantly different in mothers with frequency of CD4⁺ T cell proliferative responses $\geq 2.5\%/10^6$ PBMC to Hep B stimulation compared to those who responded poorly with T cell proliferative responses $\leq 2.5\%$. As shown in **Figure 4.10**, median of responders=0.746% versus non-responders=1.290% and $p=0.947$, and at one year postpartum median of responders=1.286% versus non-responders=1.213% and $p=0.849$.

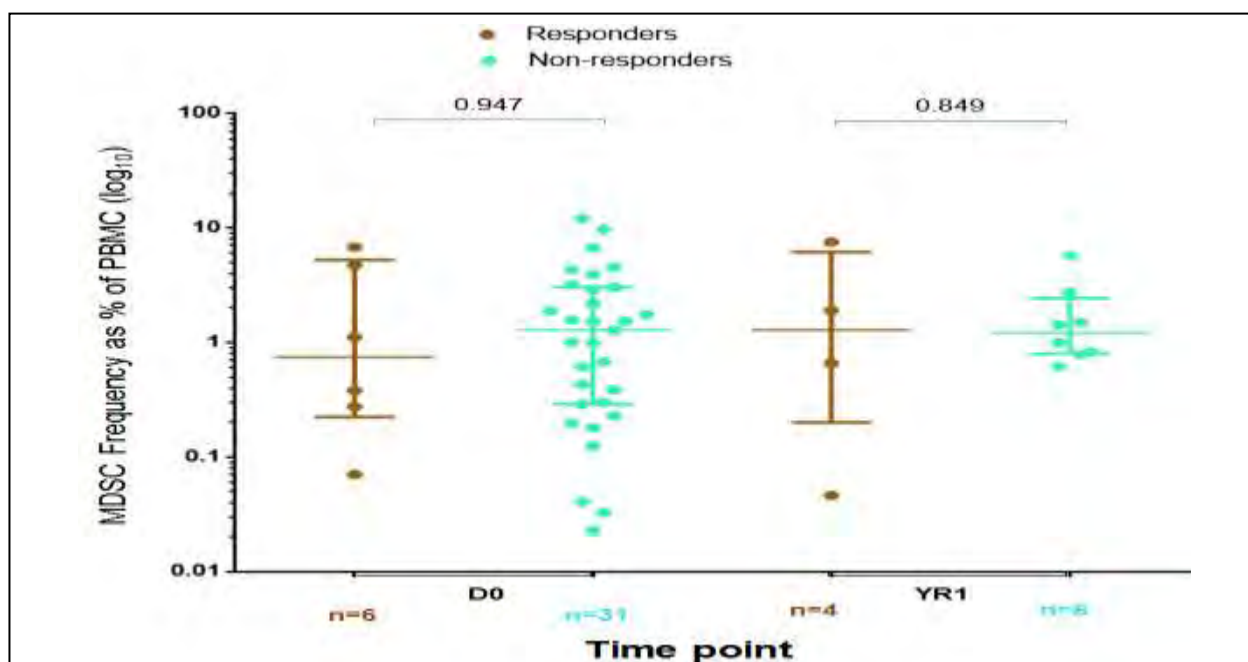
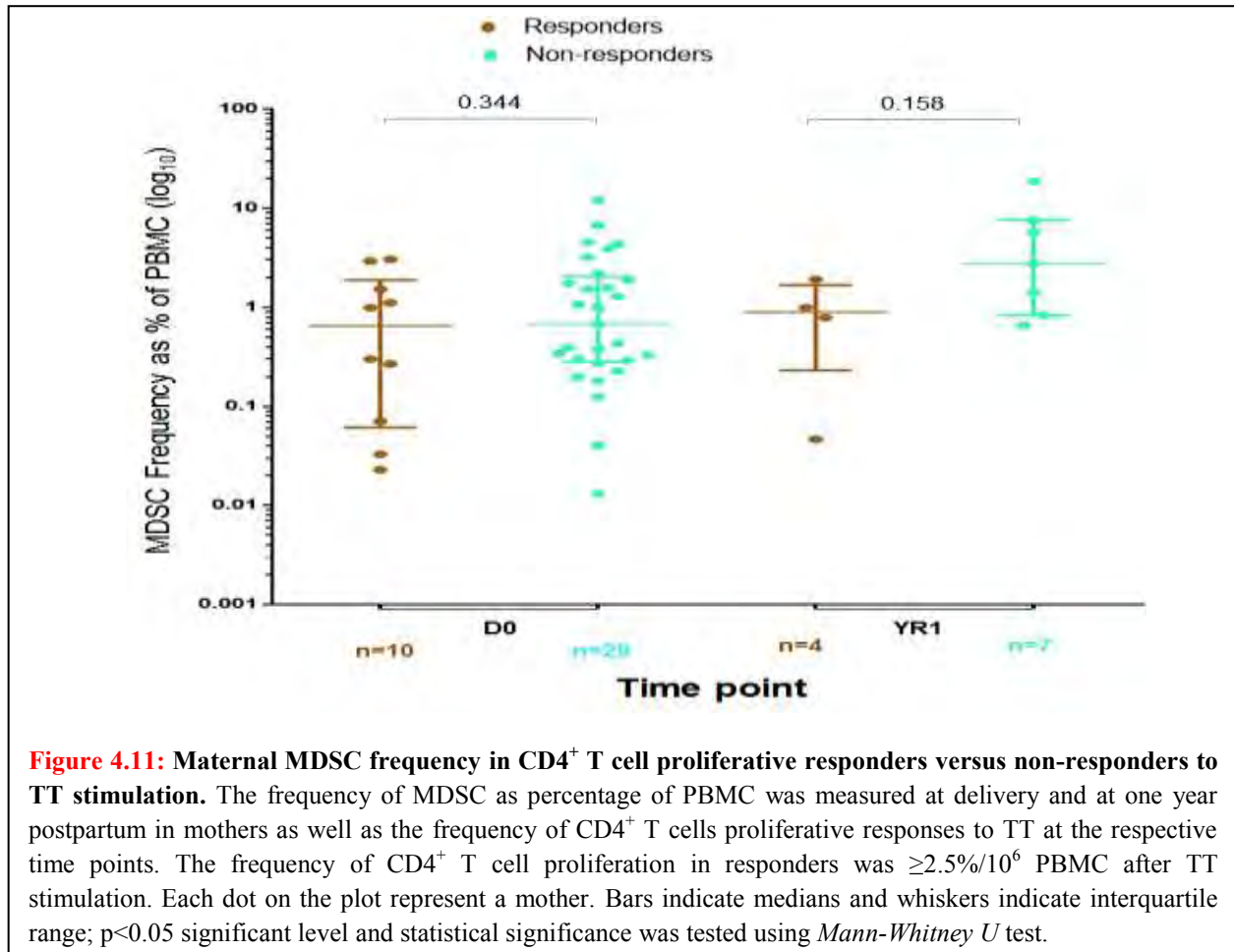


Figure 4.10: Maternal MDSC frequency in CD4⁺ T cell proliferative responders versus non-responders to Hep B stimulation. The frequency of MDSC as percentage of PBMC was measured at delivery and at one year postpartum in mothers as well as the frequency of CD4⁺ T cells proliferative responses to Hep B at the respective time points. The frequency of CD4⁺ T cell proliferation in responders was $\geq 2.5\%/10^6$ PBMC after Hep B stimulation. Each dot on the plot represent a mother. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

4.3.1.8 Maternal MDSC frequency in responders versus non-responders to TT stimulation as measured by CD4⁺ T cell proliferation

The frequency of maternal MDSC at delivery as well as at one year postpartum was not significantly different between responders and non-responders to TT stimulation. Maternal MDSC frequencies at delivery were; median of responders=0.648% versus non-responders=0.678% and $p=0.344$, and at one year postpartum were median of responders=0.892% versus non-responders=2.750% and $p=0.158$ (Figure 4.11). However, the lack of a significant difference at year one may be due to the small sample size, a significant different difference may be obtained with a larger sample size.



4.3.1.9 Maternal MDSC frequency in responders versus non-responders to PPD stimulation as measured by CD4⁺ T cell proliferation

Maternal MDSC frequency at delivery was not significantly different in mothers who responded to PPD stimulation versus those with poor CD4⁺ T cell proliferative responses to PPD at birth and at one year postpartum. As shown in Figure 4.12, maternal MDSC frequencies at delivery were median of responders=0.285% versus non-responders=0.532% and $p=0.425$, and at one year postpartum were median of responders=0.831% versus non-responders=1.500% and $p=0.260$. Also, similarly to the observation with TT, the lack of a significant difference at year one may be due to the small sample size, a significant different difference may be obtained with a larger sample size.

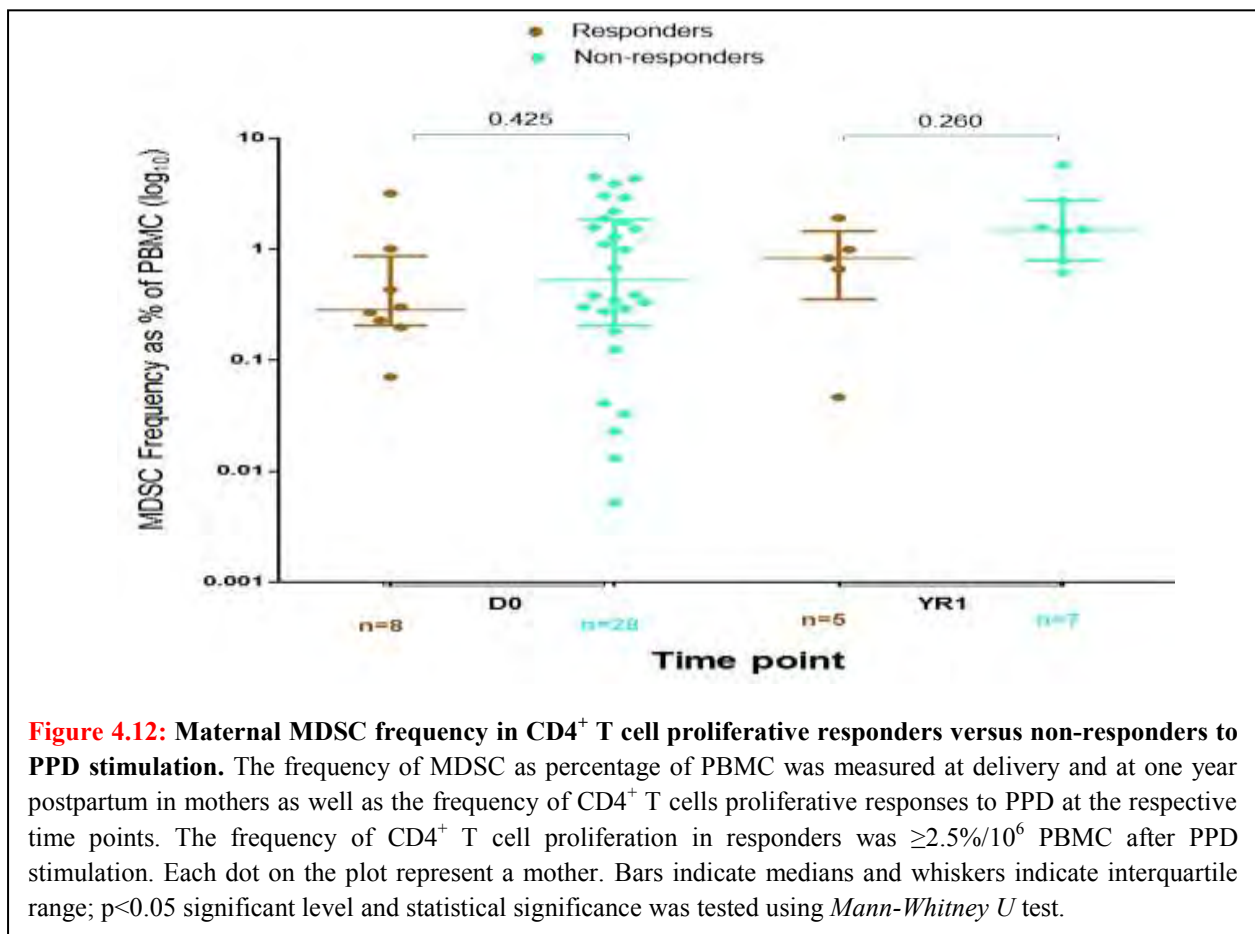


Table 4.5: Summary of the results of all infant and mothers MDSC frequency and absolute counts measured in responders versus non-responders as measured by CD4⁺ T cell proliferation. P-values of infants and mothers MDSC frequency (%) and absolute count (#) measured in responders versus non-responders as measured by CD4⁺ T cell proliferation to the following antigens [Hepatitis B (Hep B), Tetanus toxoid (TT) and Purified Protein Derivatives of *M. bovis* (PPD)]. Blue highlights represent the parameters (p-values and type of data sets), Red highlights represent the different time points [birth (D0), 6, 10 and 14 weeks (WK6, WK10 and WK14), 6 and 9 months (Mth6 and Mth9), and 1 year (YR1)]. GRP=Group, Indv.=Individual, TP= Time point, B=Baby, and M=Mother.

Parameters	p-values						
Time points	D0	WK6	WK10	WK14	Mth6	Mth9	YR1
Hep B GRP TP (%MDSC) (B)			0.767		0.143		
Hep B GRP TP (#MDSC) (B)			0.599		0.072		
TT GRP TP (%MDSC) (B)			0.606		0.966		
TT GRP TP (#MDSC) (B)			0.325		0.855		
PPD GRP TP (%MDSC) (B)			0.246		0.365		
PPD GRP TP (#MDSC) (B)			0.726		0.979		
Hep B Indv. TP (%MDSC) (M)	0.947						0.849
TT Indv. TP (%MDSC) (M)	0.344						0.158
PPD Indv. TP (%MDSC) (M)	0.425						0.260

4.3.2 CD4⁺ T cell proliferation to vaccines and antigens in PBMC (presence of MDSC) versus without MDSC (after MDSC depletion) in infants and mothers

The T cell proliferative responses to HepB and TT vaccinations and to PPD and Dynabead antigens in CBMC and/or PBMC were evaluated in infants and mothers in the presence and absence of MDSC, to evaluate the effects of MDSC more directly (Figure 4.1). Since MDSC highly express CD15, as described in section 4.2.1, the EasySep[®] magnetic cell separation technique was performed to deplete CD15⁺ expressing cells from a portion of the CBMC and/or PBMC. The effect of MDSC on T cell proliferative responses to vaccines and antigens was then measured by performing the CFSE incorporation technique on cells depleted of CD15⁺ cells (absence of MDSC) and in whole PBMC (presence of MDSC) (Figure 4.2). Among the MDSC depleted sample group, only samples with depletion purity of 90% or more were included in the analysis (Appendix 4.3).

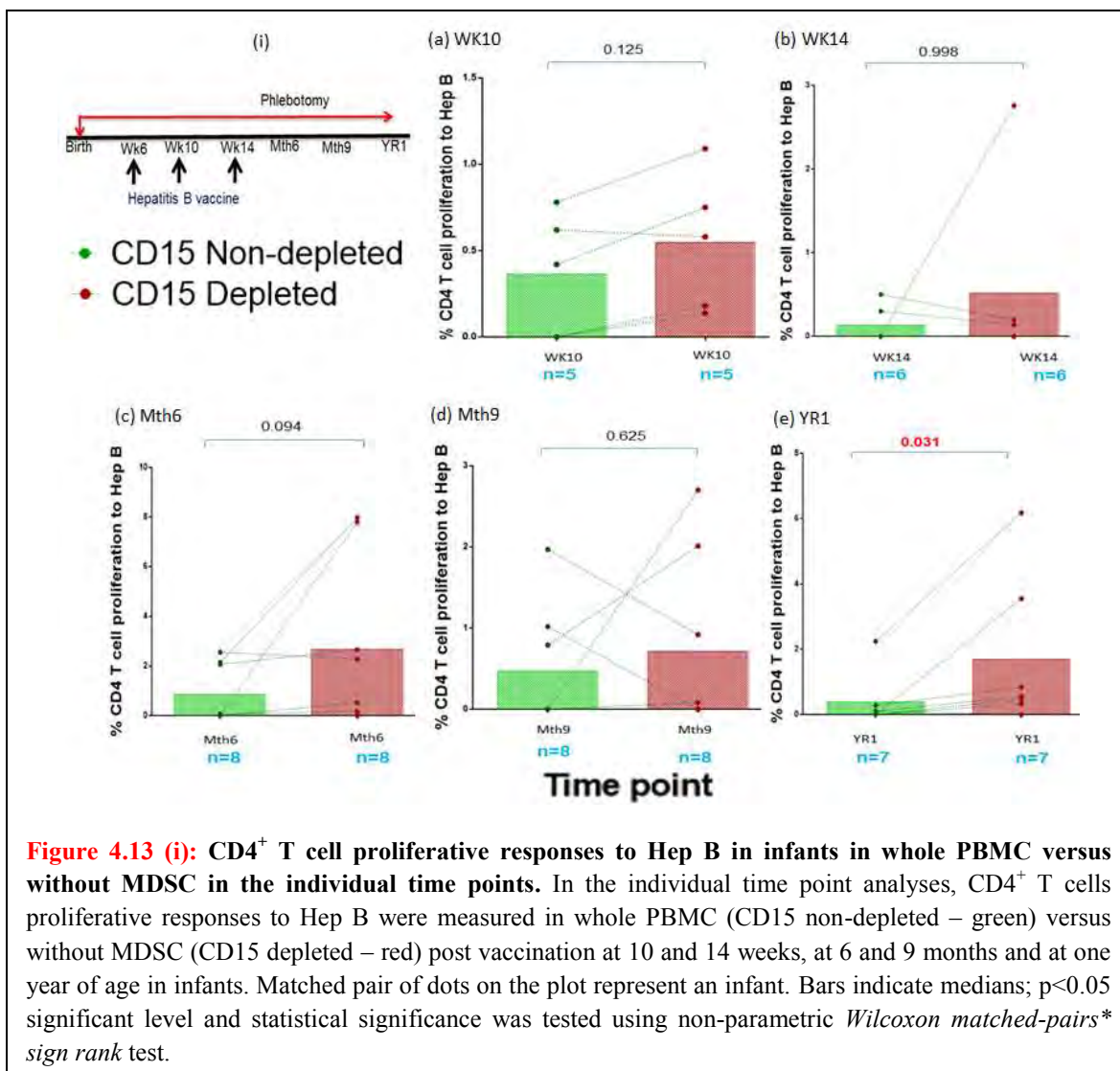
As described in section 4.3 (2), T cell proliferative responses were measured in infants at individual time points after the different vaccines were administered to infants (i.e. at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age), and in mothers after stimulation with the different antigens, T cell proliferation was measured at delivery and again at one year postpartum. We also evaluated the effect of MDSC on CD4⁺ T cell proliferation to vaccines and antigens in grouped time point analyses, based on the time points described in section 4.3 (2) (b).

The data as presented below include matched pair of samples as whole PBMC (presence of MDSC) and the absence of MDSC (MDSC depleted). Also, unmatched comparison between HEU versus HU infants as well as HIV⁺ versus HIV⁻ mothers at the respective time points either in whole PBMC (presence of MDSC) and after MDSC depletion when the number of participants were enough for statistical analyses to be performed. Table 4.6 and 4.7 below shows the summary of the results.

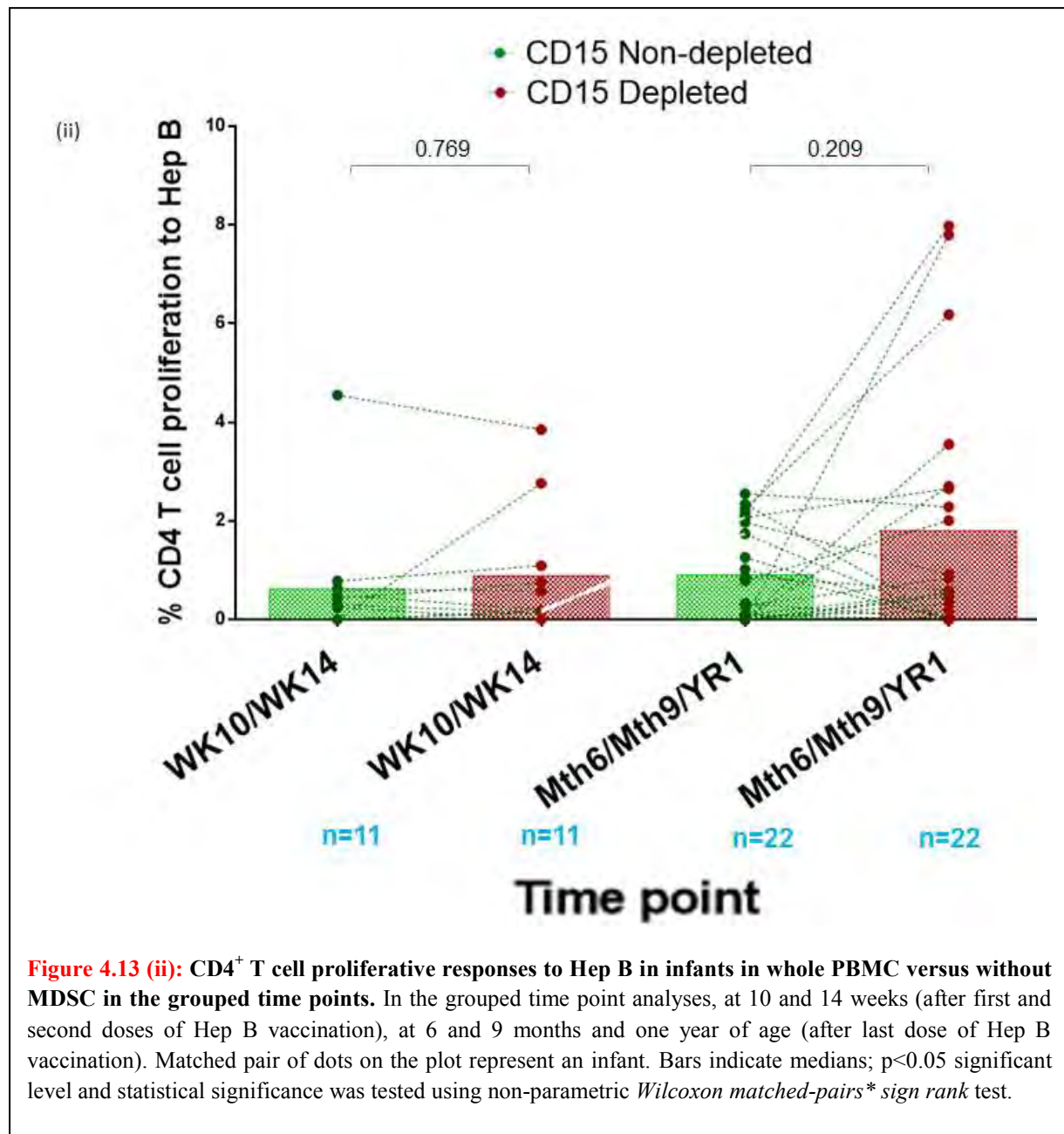
One of the challenges of this study was the large volumes of blood required for all the assays from infants, and the volume of blood collected from most infants was not always enough for CD15⁺ depletion. MDSC depletion was only performed when there was enough PBMC to perform the assays. Due to this, there were only a limited number of infants and their mothers at most time points for which depletion was performed to permit MDSC non-depleted versus MDSC depleted match paired analyses to be performed. Shown in [Appendix 4.4](#) is the unmatched analyses between MDSC non-depleted versus MDSC depleted samples in all participants enrolled and from whom blood samples were collected.

4.3.2.1 Hep B induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in infants

As shown in **Figure 4.13 (i)**, there were generally no significant differences in Hep B-induced CD4⁺ T cell proliferation at the individual time points in whole PBMC and without MDSC in infants at 10 and 14 weeks ($p=0.125$ and $p=0.998$), at 6 and 9 months ($p=0.094$ and $p=0.625$ respectively). However, at 1 year of age (median of CD15 non-depleted=0.099% versus CD15 depleted=0.550%; $p=0.031$) there were significantly higher CD4⁺ T cell proliferative responses to Hep B after MDSC depletion. There were no significant differences in the grouped time point

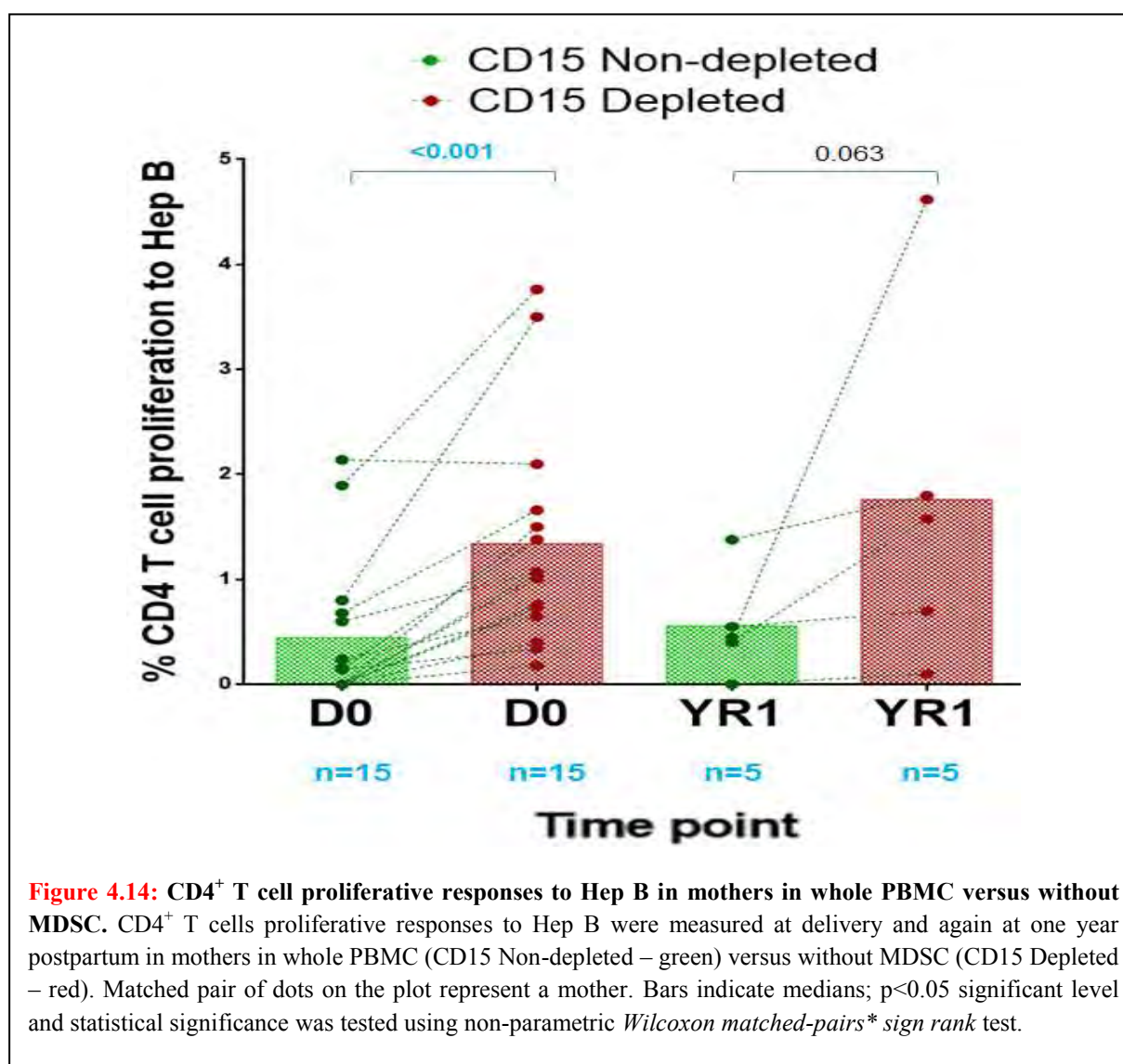


analyses in whole PBMC and without MDSC at 10 and 14 weeks (median of CD15 non-depleted=0.300% versus CD15 depleted=0.200%; $p=0.769$) and at 6, 9 months and 1 year (median of CD15 non-depleted=0.560% versus CD15 depleted=0.569%; $p=0.209$) [Figure 4.13 (ii)].



4.3.2.2 Hep B induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in mothers

In mothers, the CD4⁺ T cell proliferative responses to Hep B were evaluated in PBMC with and without MDSC at delivery and again at one year postpartum. As shown in **Figure 4.14**, there was a significantly higher CD4⁺ T cell proliferation to Hep B stimulation at delivery (median of CD15 non-depleted=0.140% versus CD15 depleted=1.050%, $p<0.001$) but this difference was not significant at one year postpartum (median of CD15 non-depleted=0.450% versus CD15 depleted=1.580%; $p=0.063$).



4.3.2.3 Hep B induced CD4⁺ T cell proliferation in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants

We compared the frequency of proliferating T cells to Hep B vaccination between HEU versus HU infants at 10 and 14 weeks; at 6 and 9 months and at one year in whole PBMC.

There were no significant differences either at the individual time points in CD4⁺ T cell proliferative responses between groups at all time points (however, at 10 weeks and at 9 months, the number of HEU infants for which Hep B proliferative responses were measured

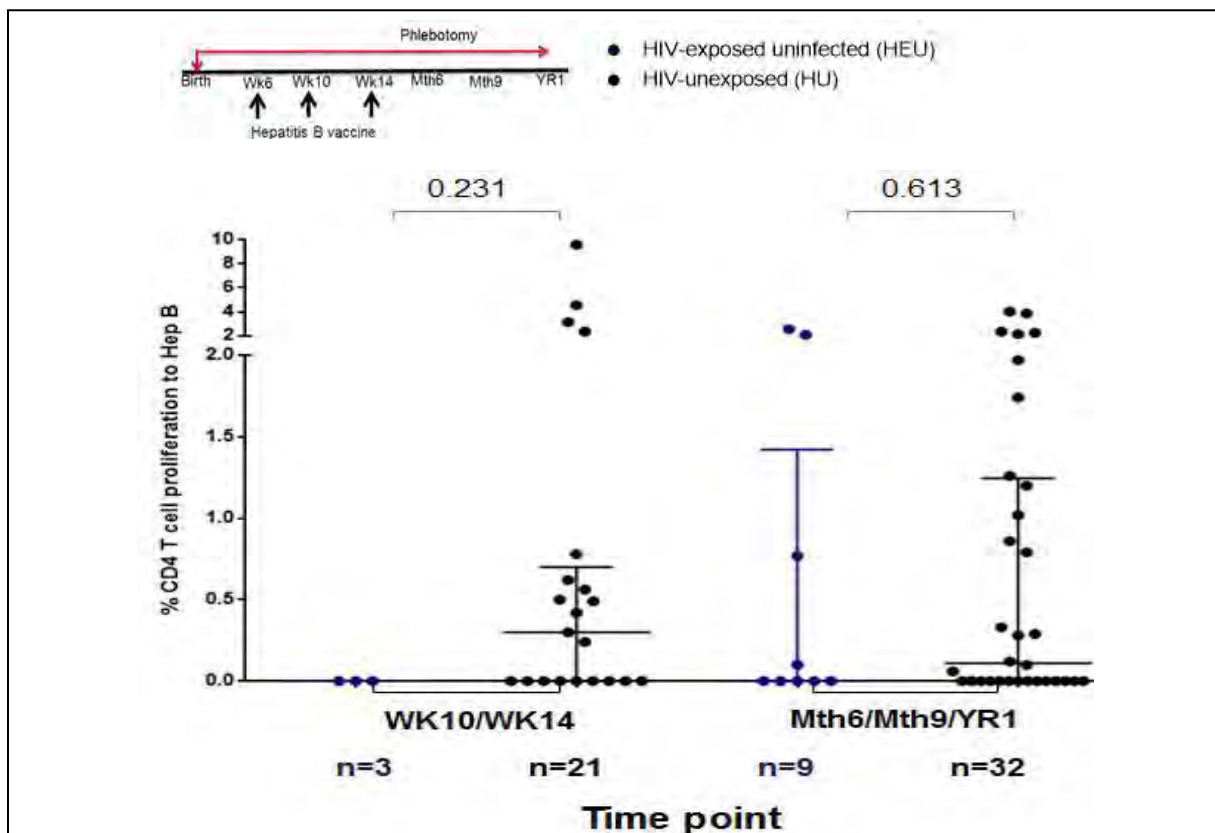
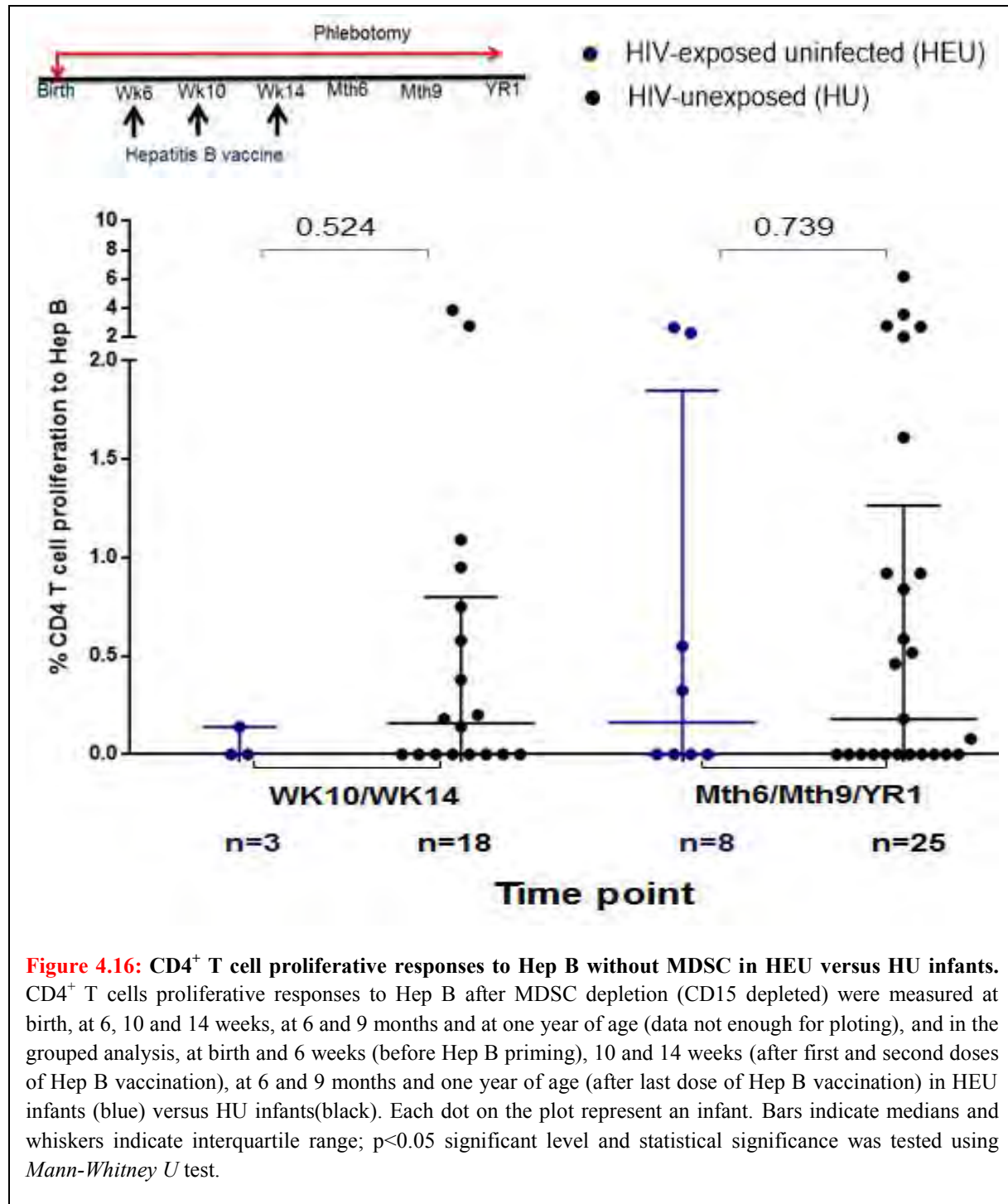


Figure 4.15: CD4⁺ T cell proliferative responses to Hep B in whole PBMC in HEU versus HU infants. CD4⁺ T cells proliferative responses to Hep B in whole PBMC (CD15 non-depleted) were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age (data not enough for plotting), and in the grouped analysis, at birth and 6 weeks (before Hep B priming), 10 and 14 weeks (after first and second doses of Hep B vaccination), at 6 and 9 months and one year of age (after last dose of Hep B vaccination) in HEU infants (blue) versus HU infants (black). Each dot on the plot represents an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using Mann-Whitney U test.

was small and statistical analysis could not be performed; data not shown). In the grouped analyses, there was no significant difference in proliferative response in the HEU versus HU infants at 10 and 14 weeks grouped time points (median of HEU=0% versus HU=0.490%; $p=0.231$) and at 6, 9 months and 1 year (median of HEU=0.102% versus HU=0.40%; $p=0.613$) (Figure 4.15).

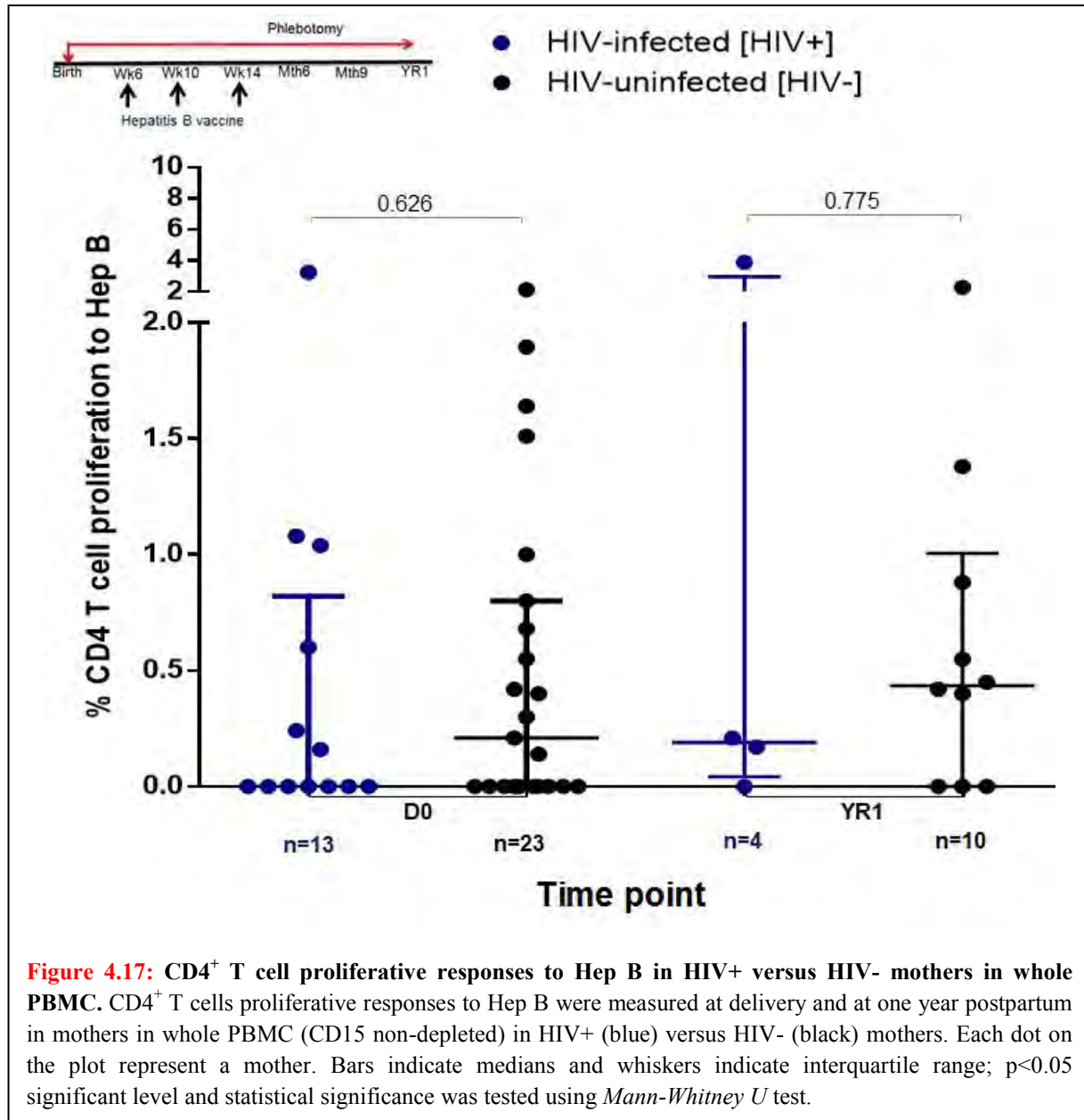
4.3.2.4 Hep B induced CD4⁺ T cell proliferation after MDSC depletion in HEU versus HU infants

Although we found no statistically significant differences in MDSC frequency in HEU vs HU infants (Chapter 3, section 3.3.1.2) even though there was a trend towards a high MDSC frequency in HEU infants, we evaluated if HIV-exposure would alter the functional effect of MDSC on CD4⁺ T cell proliferation. We compared the frequency of proliferating T cells to Hep B after MDSC depletion between HEU versus HU infants at 10 and 14 weeks; at 6 and 9 months and at one year of age. There were no significant differences at the individual time points in CD4⁺ T cell proliferative responses between groups (however, at 10 weeks and at 9 months, the number of HEU infants for which Hep B proliferative responses were measured was small and statistical analysis could not be performed; data not shown). In the grouped time point analyses at 10 and 14 weeks (median of HEU=0.001% versus HU=0.380%; $p=0.524$) and at 6, 9 months and 1 year (median of HEU=0.275% versus HU=0.2981%; $p=0.739$) there were no significant differences observed (Figure 4.16).



4.3.2.5 Hep B induced CD4⁺ T cell proliferation in HIV+ versus HIV- mothers

We compared the frequency of proliferative T cell responses to Hep B in PBMC between HIV+ versus HIV- mothers at delivery and at one year postpartum. As shown in **Figure 4.17**, there were no significant differences in CD4⁺ T cell proliferative responses between groups either at delivery or at one year postpartum.



4.3.2.6 Hep B induced CD4⁺ T cell proliferation in HIV+ versus HIV- mothers after MDSC depletion

We compared the effect of MDSC depletion on CD4⁺ T cell proliferation to Hep B in HIV-infected versus negative mothers at delivery and at one year postpartum. Depleting MDSC had little effect on CD4⁺ T cell proliferative responses between groups at all time points (however, at 1 year postpartum the number of HIV+ mothers for which Hep B proliferative responses were measured was small and statistical analysis could not be performed; data not shown).

4.3.2.7 TT induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in infants

The CD4⁺ T cell proliferative responses to TT vaccination were evaluated in whole PBMC and without MDSC in infants at all time points post TT vaccination in infant [Figure 4.18 (i)]. We compared CD4⁺ T cell proliferative responses at the individual time points after TT

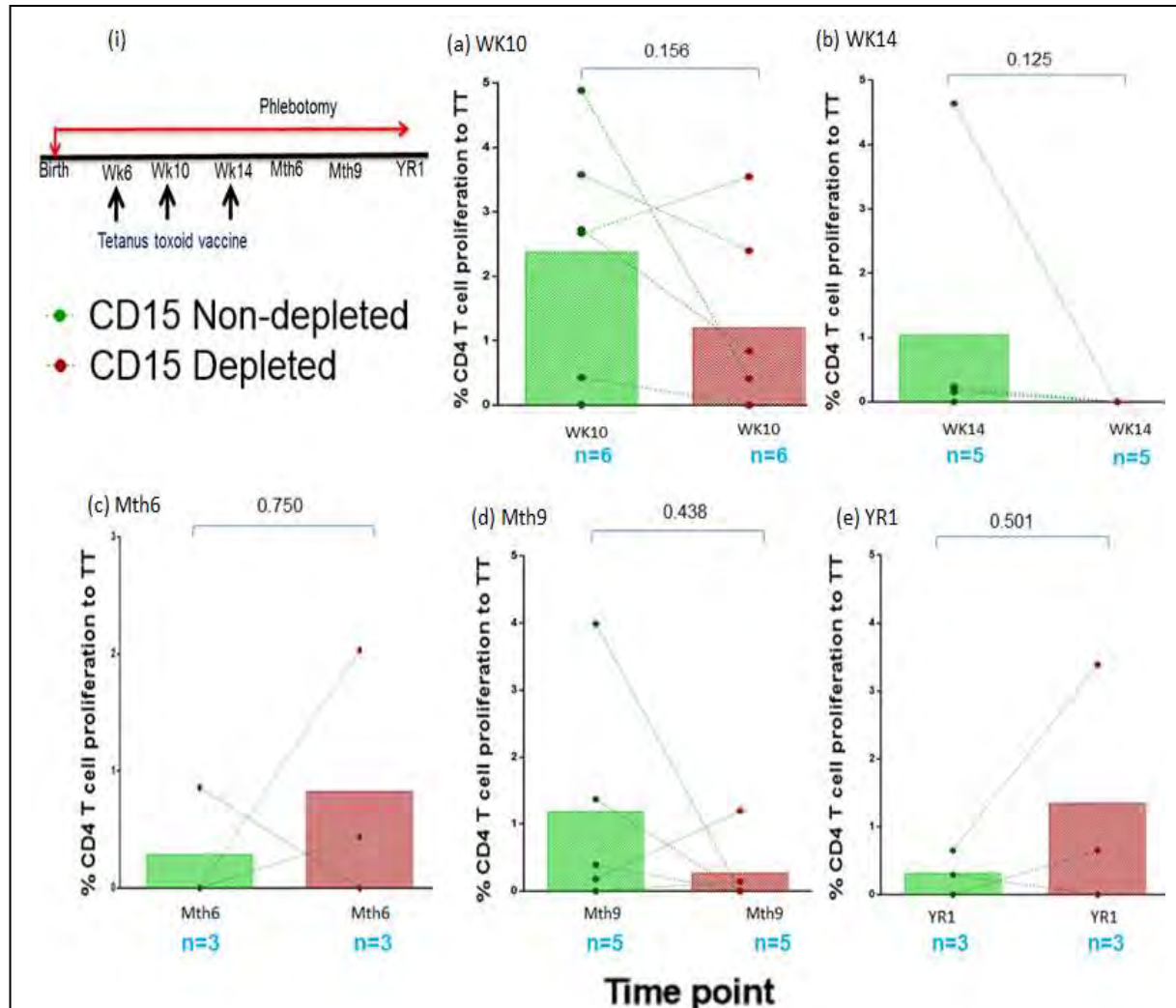
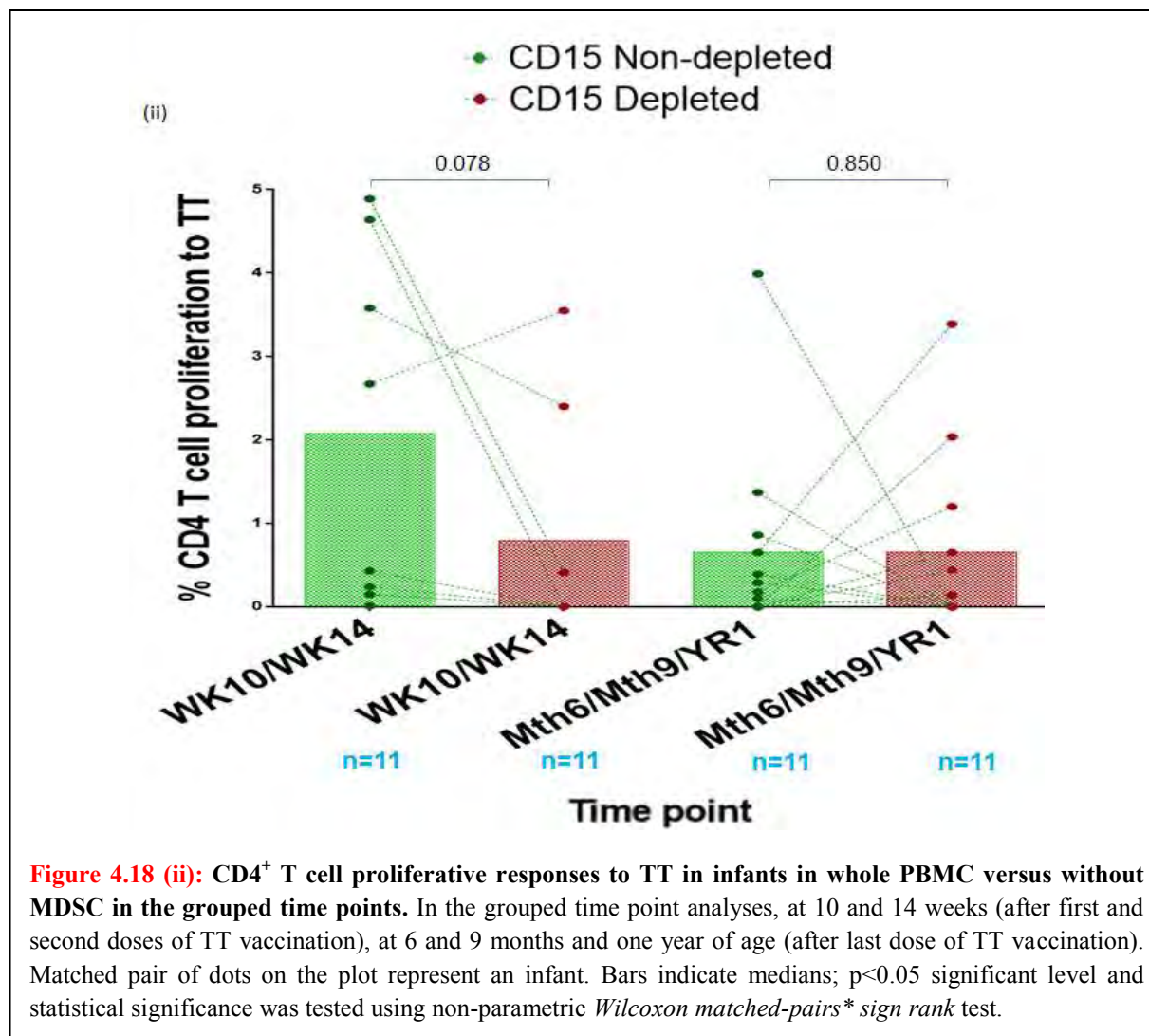


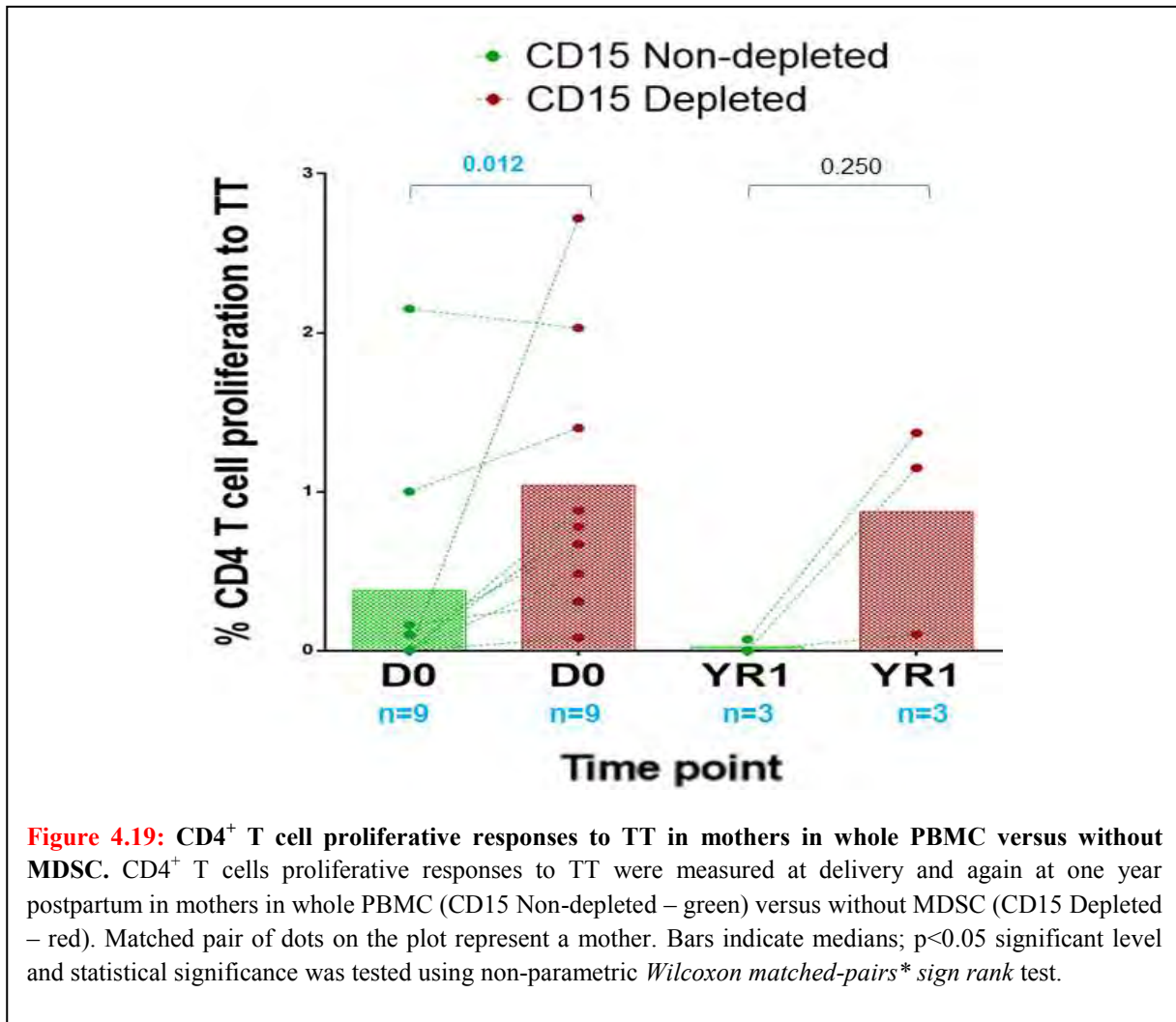
Figure 4.18 (i): CD4⁺ T cell proliferative responses to TT in infants in whole PBMC versus without MDSC in the individual time points. In the individual time point analyses, CD4⁺ T cells proliferative responses to TT were measured in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red) post vaccination at 10 and 14 weeks, at 6 and 9 months and at one year of age in infants. Matched pair of dots on the plot represent an infant. Bars indicate medians; $p < 0.05$ significant level and statistical significance was tested using non-parametric *Wilcoxon matched-pairs* sign rank* test.

vaccination between whole PBMC and without MDSC in infants. There was no significant difference at 10 and 14 weeks ($p=0.156$ and $p=0.125$ respectively), at 6 and 9 months ($p=0.750$ and $p=0.438$ respectively) and at one year ($p=0.501$) of age. In the grouped time point analyses in whole PBMC versus without MDSC, there was no significant difference in $CD4^+$ T cell proliferative responses to TT at 10 and 14 weeks ($p=0.078$), and at 6, 9 months and one year ($p=0.850$) [Figure 4.18 (ii)].



4.3.2.8 TT induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in mothers

In mothers, the CD4⁺ T cell proliferative responses to TT were evaluated in whole PBMC and without MDSC at delivery and again at one year postpartum. As shown in **Figure 4.19**, there was a significantly higher maternal CD4⁺ T cell proliferative responses to TT stimulation at delivery after MDSC depletion (median of CD15 non-depleted=0%, IQR=0-0.580 versus CD15 depleted=0.780%, IQR=0.397-1.715 and $p=0.012$) but not at one year postpartum (median of CD15 non-depleted=0%, IQR=0-0.070 versus CD15 depleted=0%, IQR=0.101-1.370 and $p=0.250$).



4.3.2.9 TT induced CD4⁺ T cell proliferation in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants

We compared the frequency of proliferating CD4⁺ T cells to TT vaccination in whole PBMC between HEU versus HU infants at all time points post TT vaccination. There were no significant differences in CD4⁺ T cell proliferative responses between groups at any time point either, (a) at the individual time points or (b) in the grouped analysis (Figure 4.20).

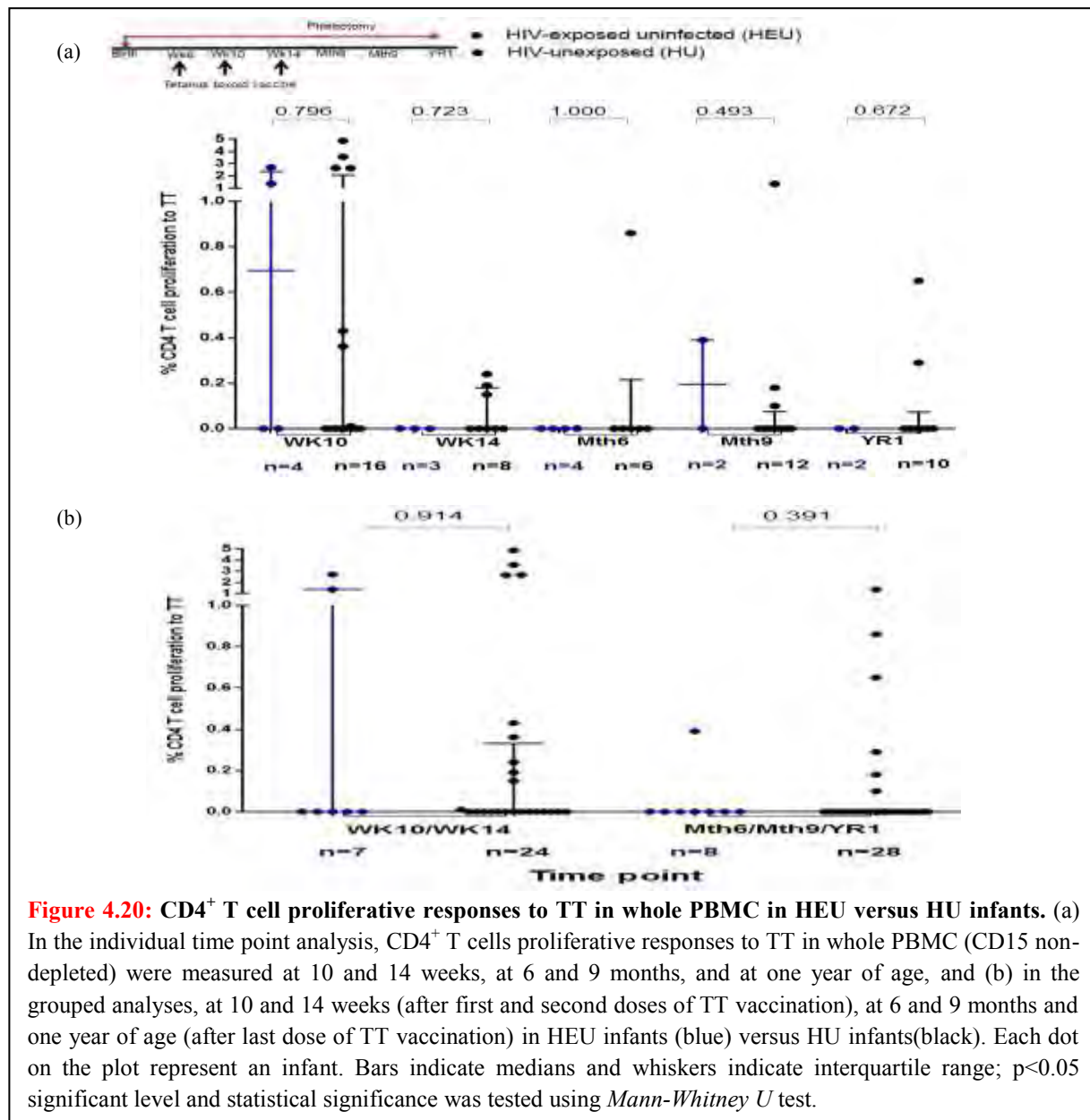
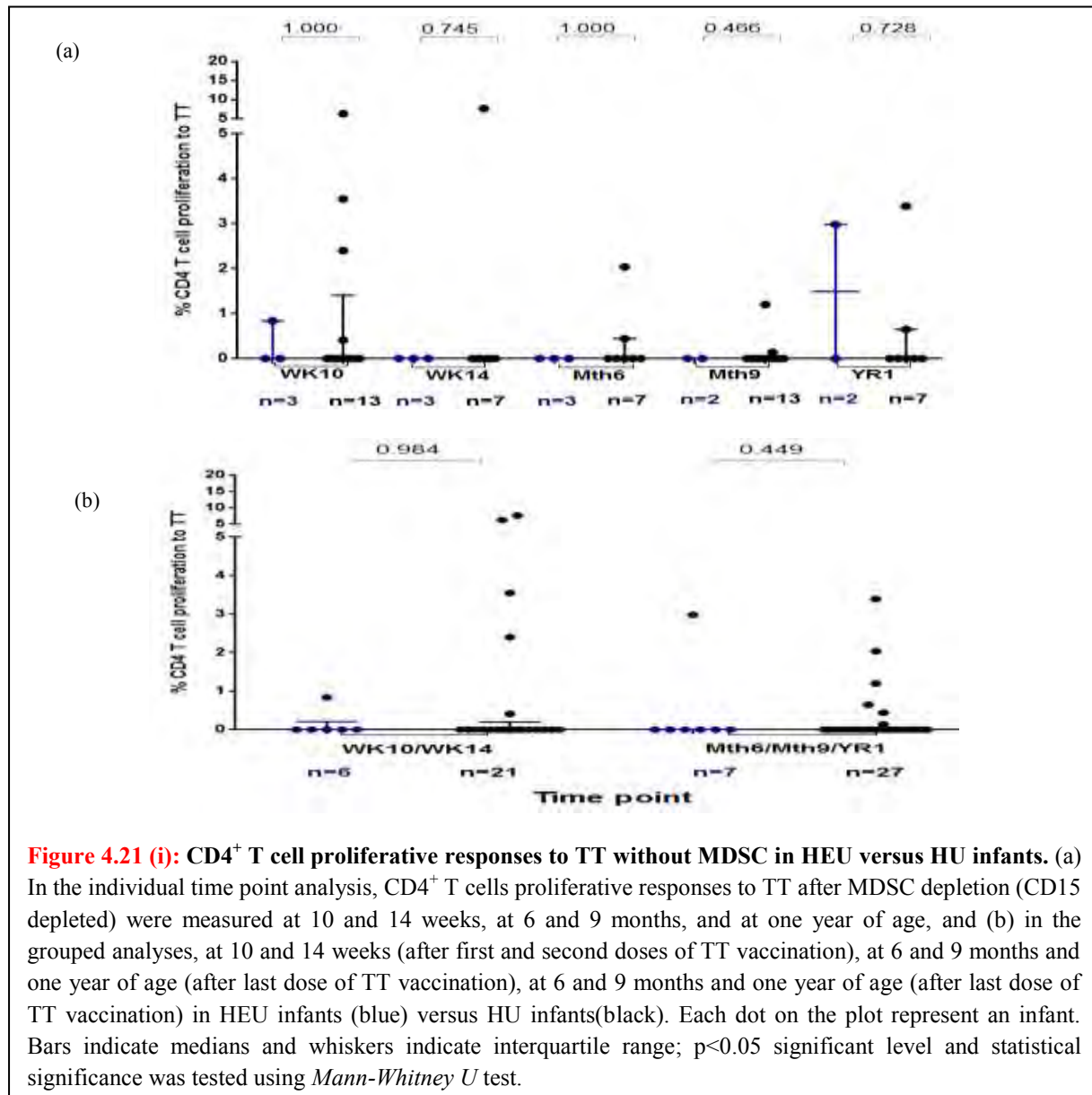


Figure 4.20: CD4⁺ T cell proliferative responses to TT in whole PBMC in HEU versus HU infants. (a) In the individual time point analysis, CD4⁺ T cells proliferative responses to TT in whole PBMC (CD15 non-depleted) were measured at 10 and 14 weeks, at 6 and 9 months, and at one year of age, and (b) in the grouped analyses, at 10 and 14 weeks (after first and second doses of TT vaccination), at 6 and 9 months and one year of age (after last dose of TT vaccination) in HEU infants (blue) versus HU infants (black). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; p<0.05 significant level and statistical significance was tested using *Mann-Whitney U* test.

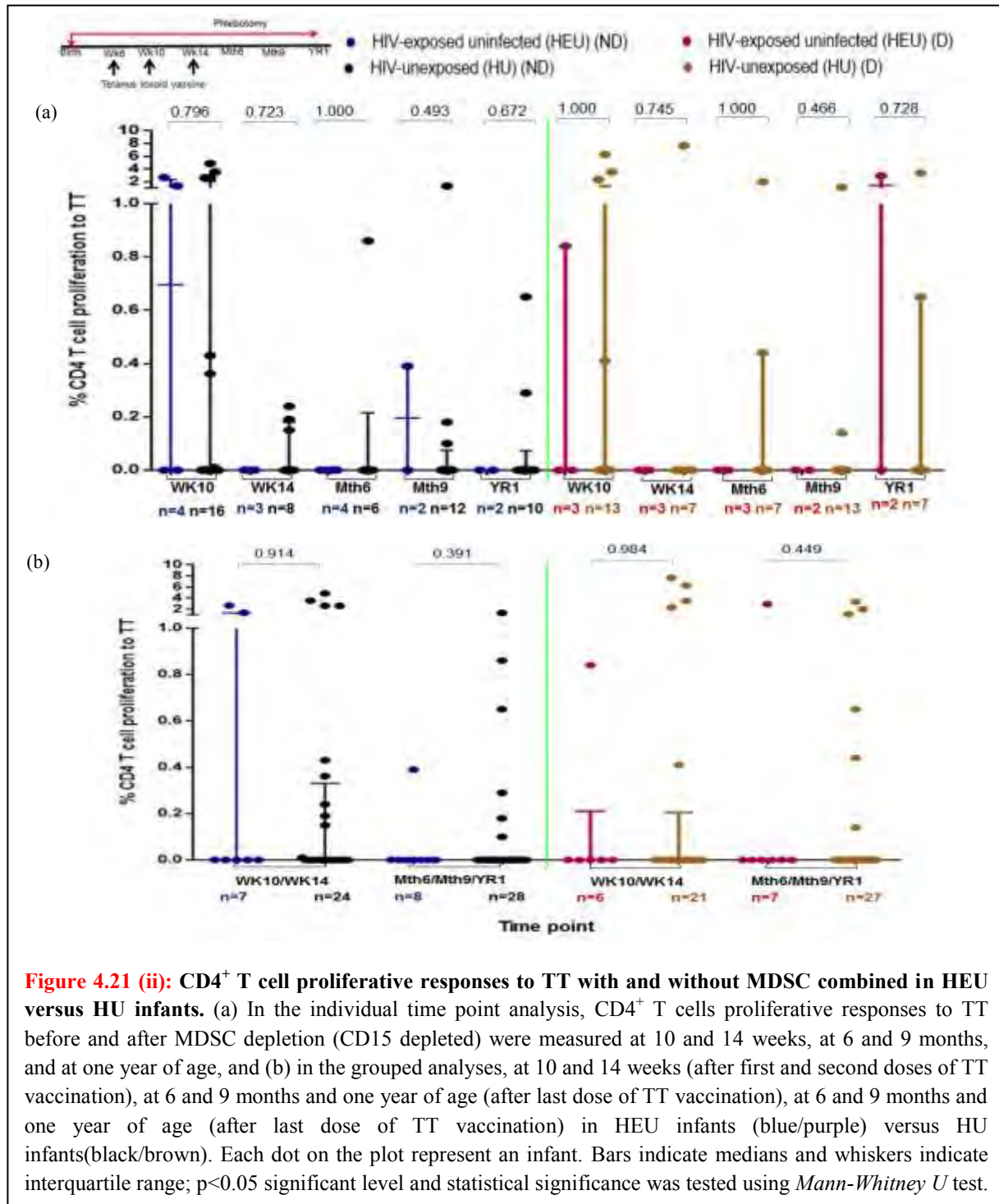
4.3.2.10 TT induced CD4⁺ T cell proliferation after MDSC depletion in HEU versus HU infants

To evaluate if HIV-exposure would contribute to the effect of MDSC on CD4⁺ T cell proliferation, we compared the frequency of proliferative responses to TT vaccination in the



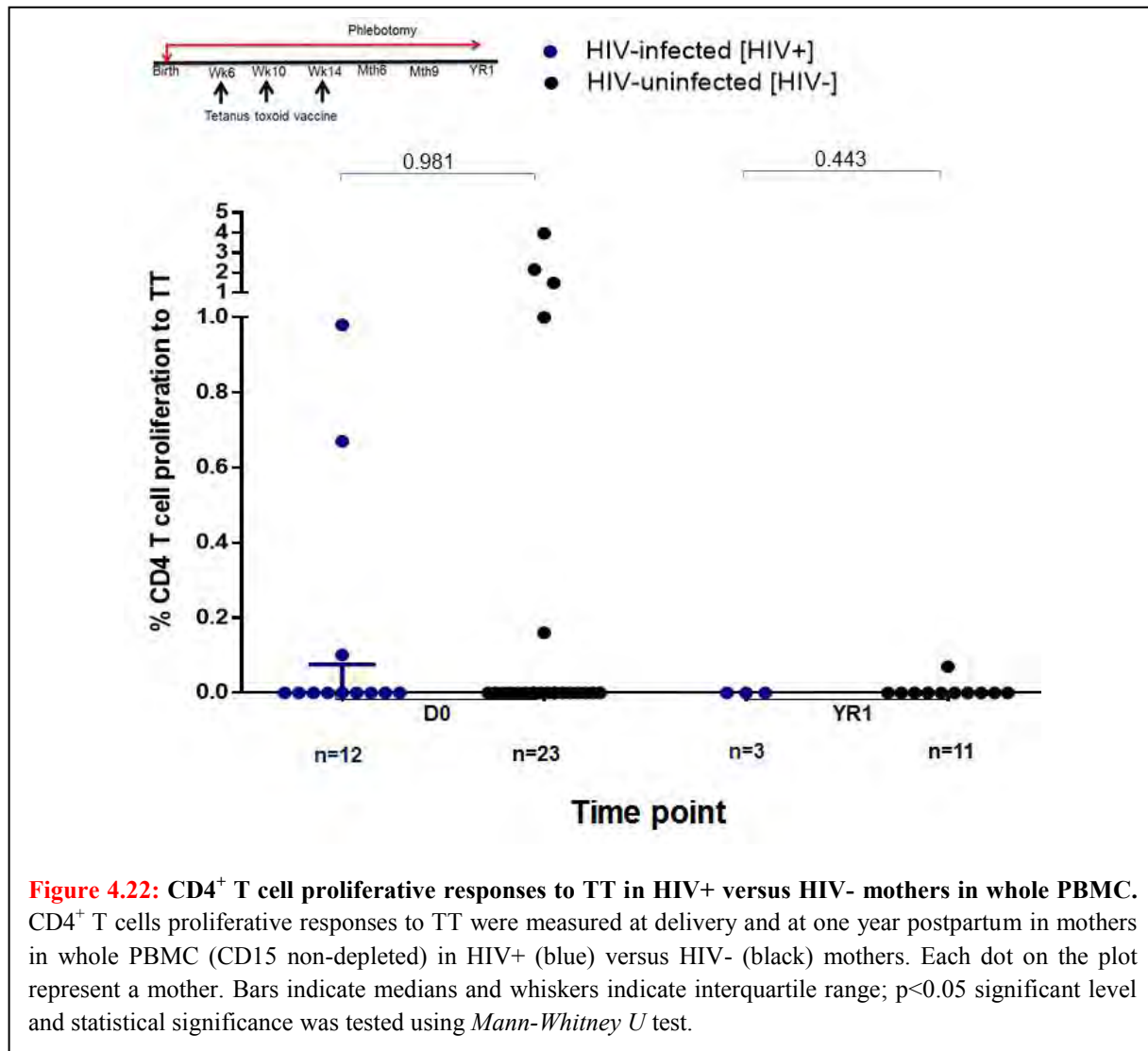
absence of MDSC between HEU versus HU infants in the first year of life. As shown in Figure 4.21 (i), there were no significant differences in CD4⁺ T cell proliferative responses between groups at all time points either, (a) at the individual time points or (b) in the grouped analysis.

Further, Figure 4.21 (ii) shows a combination of CD4⁺ T cell proliferative responses to TT with and without MDSC in HEU vs HU infants.



4.3.2.11 TT induced CD4⁺ T cell proliferation in HIV+ versus HIV- mothers

We evaluated the frequency of proliferating T cells to TT between HIV+ versus HIV- mothers at delivery and at one year postpartum. As shown in [Figure 4.22](#), there were no significant differences in CD4⁺ T cell proliferative responses between groups at all time points. However, responses were very low for both groups.



4.3.2.12 TT induced CD4⁺ T cell proliferation in HIV+ versus HIV- mothers after MDSC depletion

We also evaluated if depleting MDSC would have different effects in HIV-infected versus uninfected mothers on CD4⁺ T cell proliferation to TT at delivery and at one year postpartum. There were no significant differences in CD4⁺ T cell proliferative responses between groups at all-time points. However, responses were very low for both groups and at 1 year postpartum the number of HIV+ mothers for which TT proliferative responses were measured was small and statistical analysis could not be performed; data not shown).

4.3.2.13 PPD induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in infants

The CD4⁺ T cell proliferative responses to *Mycobacterium bovis* BCG vaccination were evaluated in whole PBMC and without MDSC in infants at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age, by measuring responses to PPD stimulation.

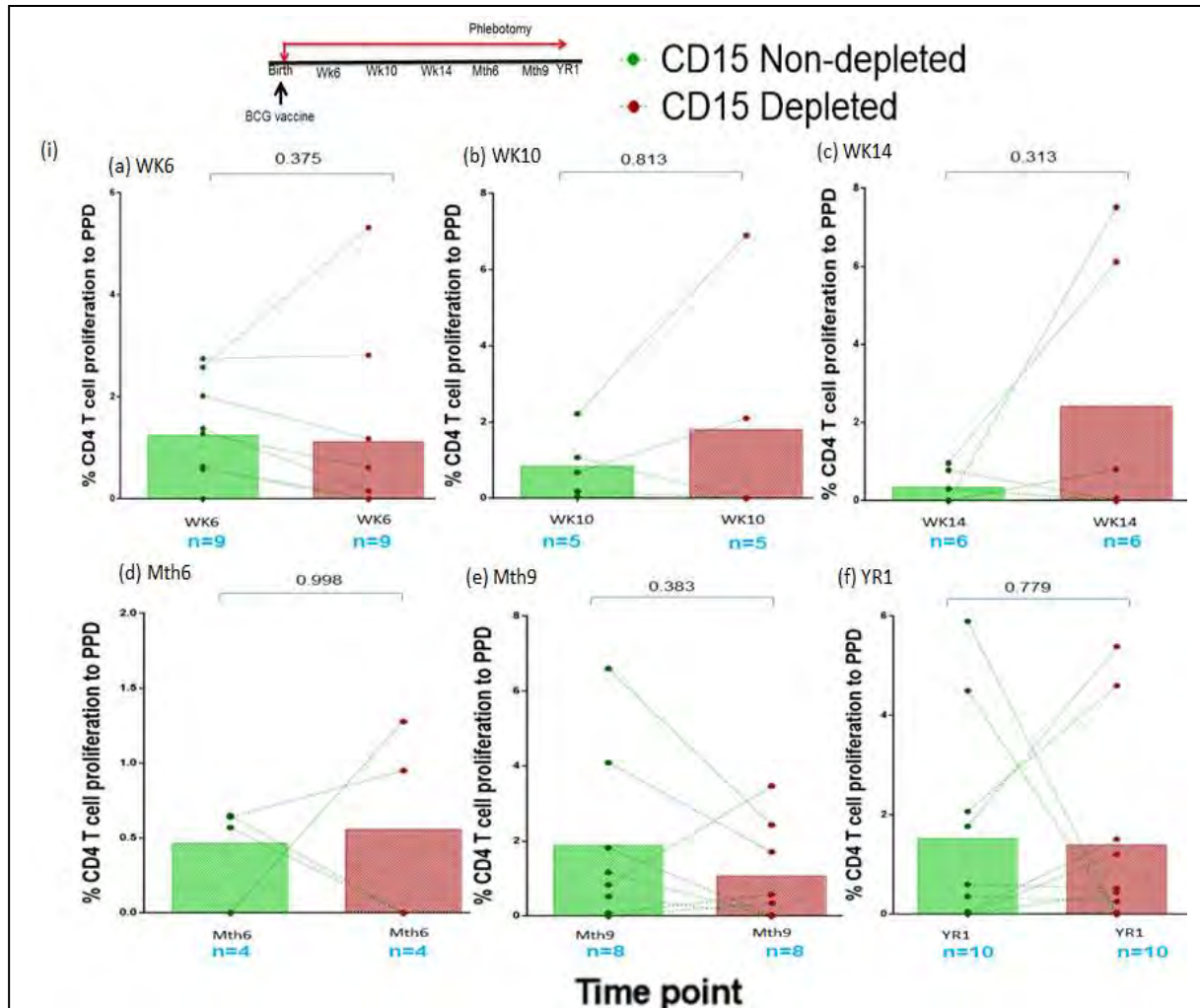
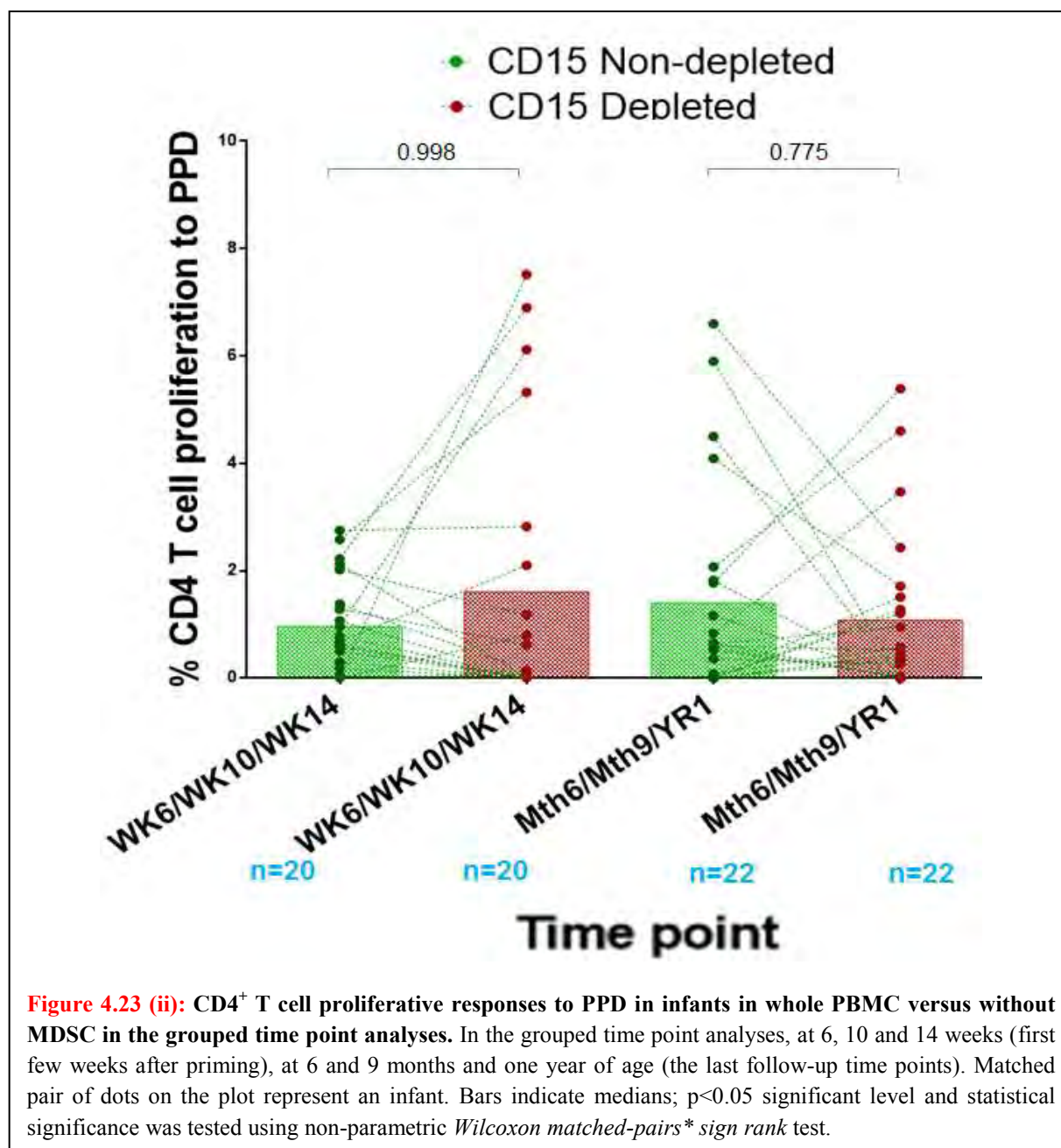


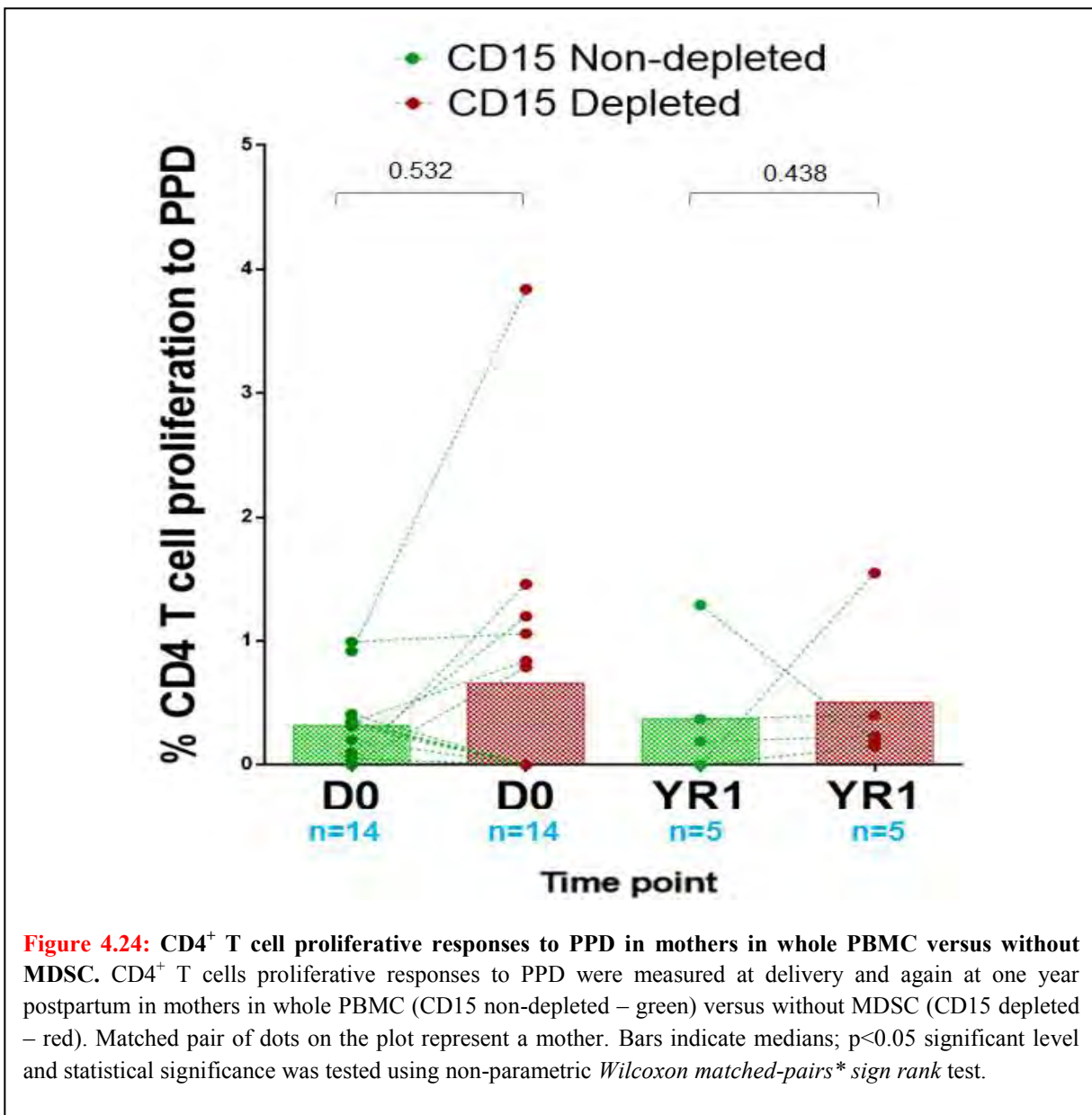
Figure 4.23 (i): CD4⁺ T cell proliferative responses to PPD in infants in whole PBMC versus without MDSC in the individual time points. In the individual time point analyses, CD4⁺ T cells proliferative responses to PPD were measured in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red) at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants. Matched pair of dots on the plot represent an infant. Bars indicate medians; p<0.05 significant level and statistical significance was tested using non-parametric *Wilcoxon matched-pairs* sign rank* test.

There were no significant differences either at the individual time points at 6, 10 and 14 weeks ($p=0.375$, $p=0.813$ and $p=0.313$ respectively), at 6 and 9 months ($p=0.996$ and $p=0.383$ respectively) and at one year ($p=0.779$) [(Figure 4.23) (i)]. In the grouped time point analyses there was no significant difference with and without MDSC at 6, 10 and 14 weeks ($p=0.998$) and at 6, 9 months and one year ($p=0.775$) [(Figure 4.23) (ii)].



4.3.2.14 PPD induced $CD4^+$ T cell proliferation in total PBMC versus after MDSC depletion in mothers

In mothers, $CD4^+$ T cell proliferative responses to PPD were evaluated in PBMC before and after MDSC depletion at delivery and again at one year postpartum. As shown in Figure 4.24, there was no significant difference at delivery ($p=0.532$) and at one year postpartum ($p=0.438$) (Figure 4.24).



4.3.2.15 PPD induced CD4⁺ T cell proliferation in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants

We compared the frequency of proliferating T cells to PPD stimulation between HEU versus HU infants at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in whole PBMC.

As shown in **Figure 4.25**, there were no significant differences in CD4⁺ T cell proliferative responses between groups at any time point either, (a) at the individual time points or (b) in the grouped analysis.

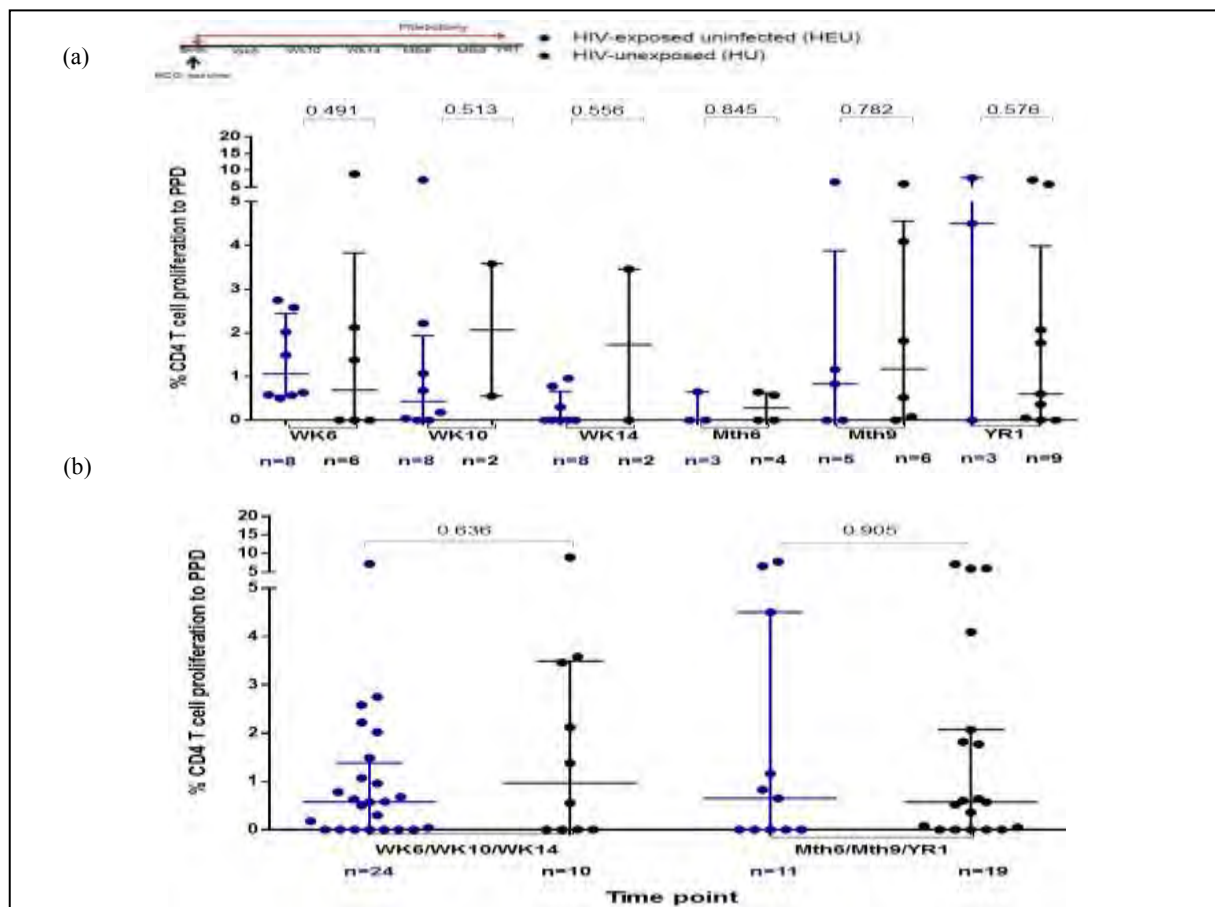


Figure 4.25: CD4⁺ T cell proliferative responses to PPD in whole PBMC in HEU versus HU infants. (a) In the individual time point analysis, CD4⁺ T cells proliferative responses to PPD in whole PBMC (CD15 non-depleted) were measured at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age, and (b) in the grouped analysis, at 6, 10 and 14 weeks (first few weeks after priming), at 6 and 9 months and one year of age (the last follow-up time points) in HEU infants (blue) versus HU infants (black). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

4.3.2.16 PPD induced CD4⁺ T cell proliferation after MDSC depletion in HEU versus HU infants

To evaluate if HIV-exposure would influence the effect of MDSC on CD4⁺ T cell proliferation to PPD, we compared the frequency of proliferating T cells to PPD stimulation after MDSC depletion between HEU versus HU infants at all time points (Figure 4.26). There were no significant differences in CD4⁺ T cell proliferative responses between groups at all time points either, at the individual time points (however, at 10 and 14 weeks, the number of HU infants for which Hep B proliferative responses were measured was small and statistical analysis could not be performed; data not shown), or in the grouped analysis. Further, Figure 4.26 (ii) shows a combination of CD4⁺ T cell proliferative responses to PPD with and without MDSC in HEU vs HU infants.

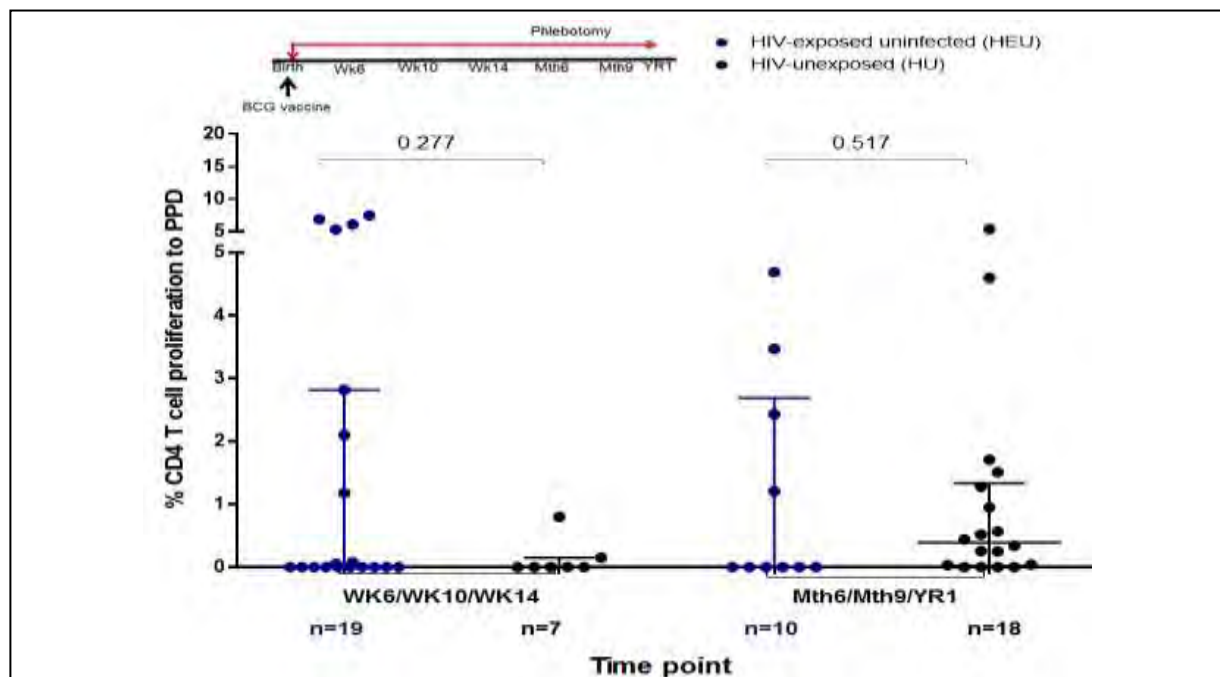


Figure 4.26 (i): CD4⁺ T cell proliferative responses to PPD without MDSC in HEU versus HU infants. CD4⁺ T cells proliferative responses to PPD without MDSC (CD15 depleted) were measured at 6, 10 and 14 weeks, at 6 and 9 months and at one year year of age (data not enough for plotting), and in the grouped analysis, at 6, 10 and 14 weeks (first few weeks after priming), at 6 and 9 months and one year of age (the last follow-up time points) in HEU infants (blue) versus HU infants (black). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

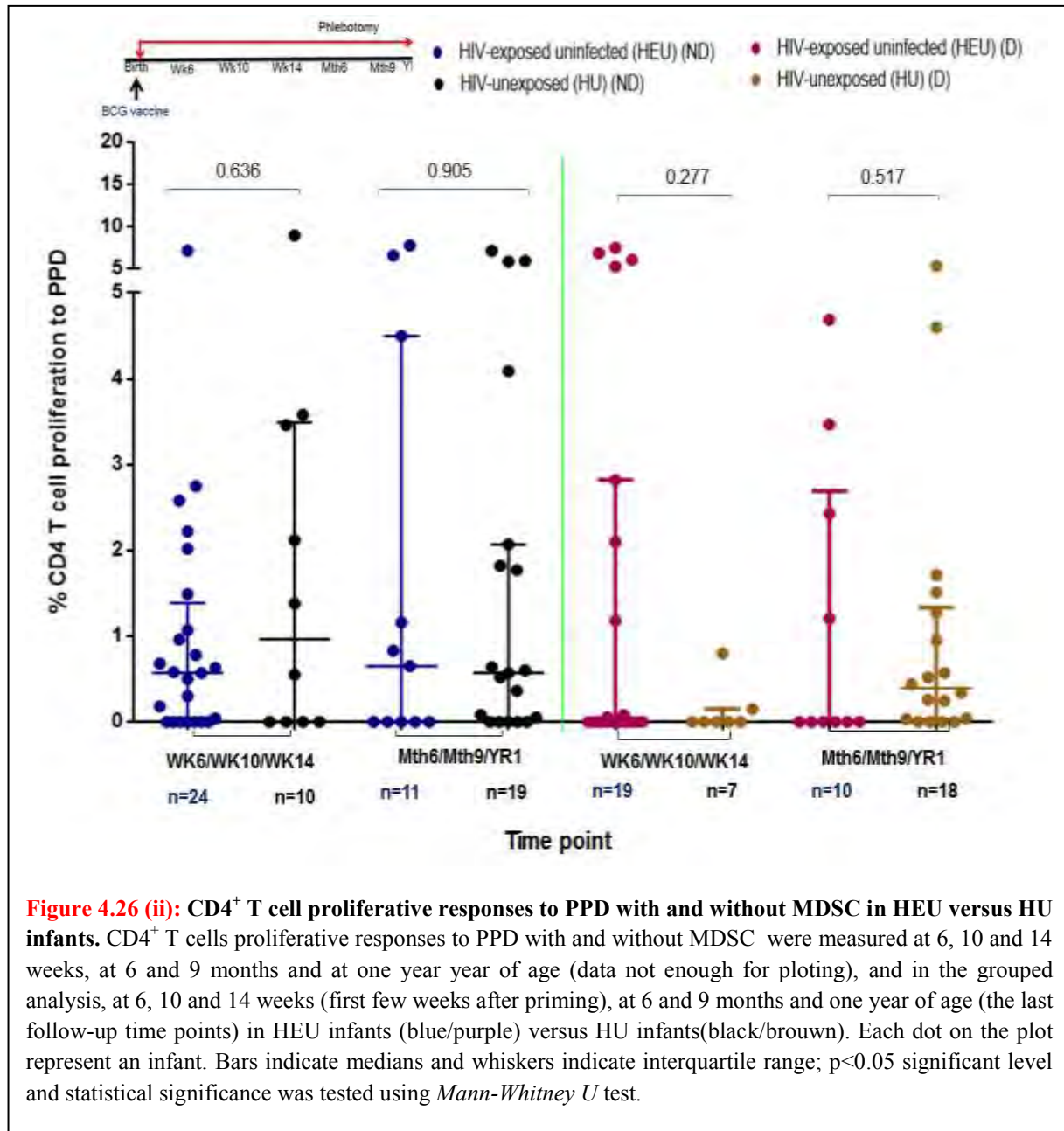
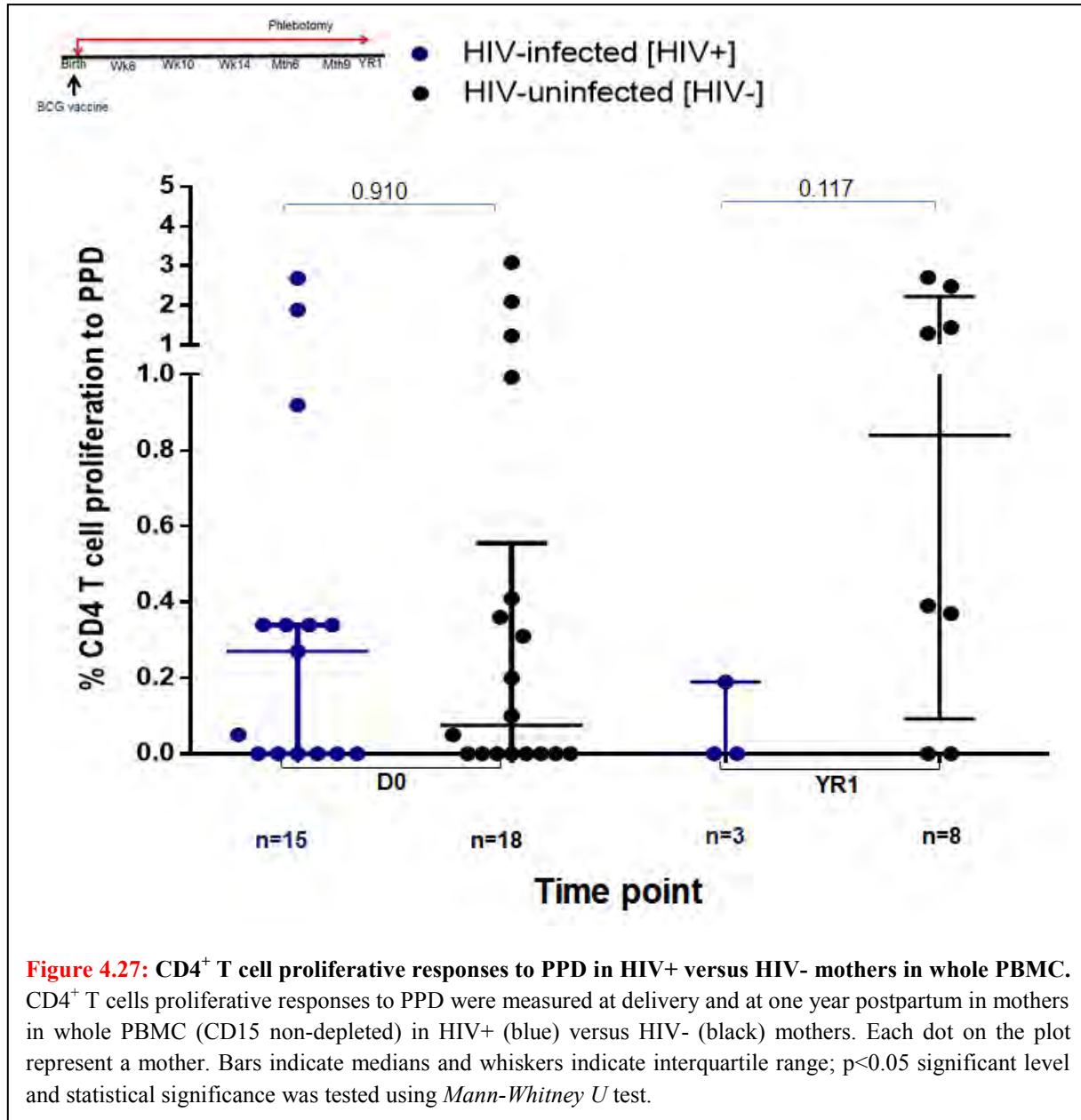


Figure 4.26 (ii): CD4⁺ T cell proliferative responses to PPD with and without MDSC in HEU versus HU infants. CD4⁺ T cells proliferative responses to PPD with and without MDSC were measured at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age (data not enough for plotting), and in the grouped analysis, at 6, 10 and 14 weeks (first few weeks after priming), at 6 and 9 months and one year of age (the last follow-up time points) in HEU infants (blue/purple) versus HU infants (black/brown). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

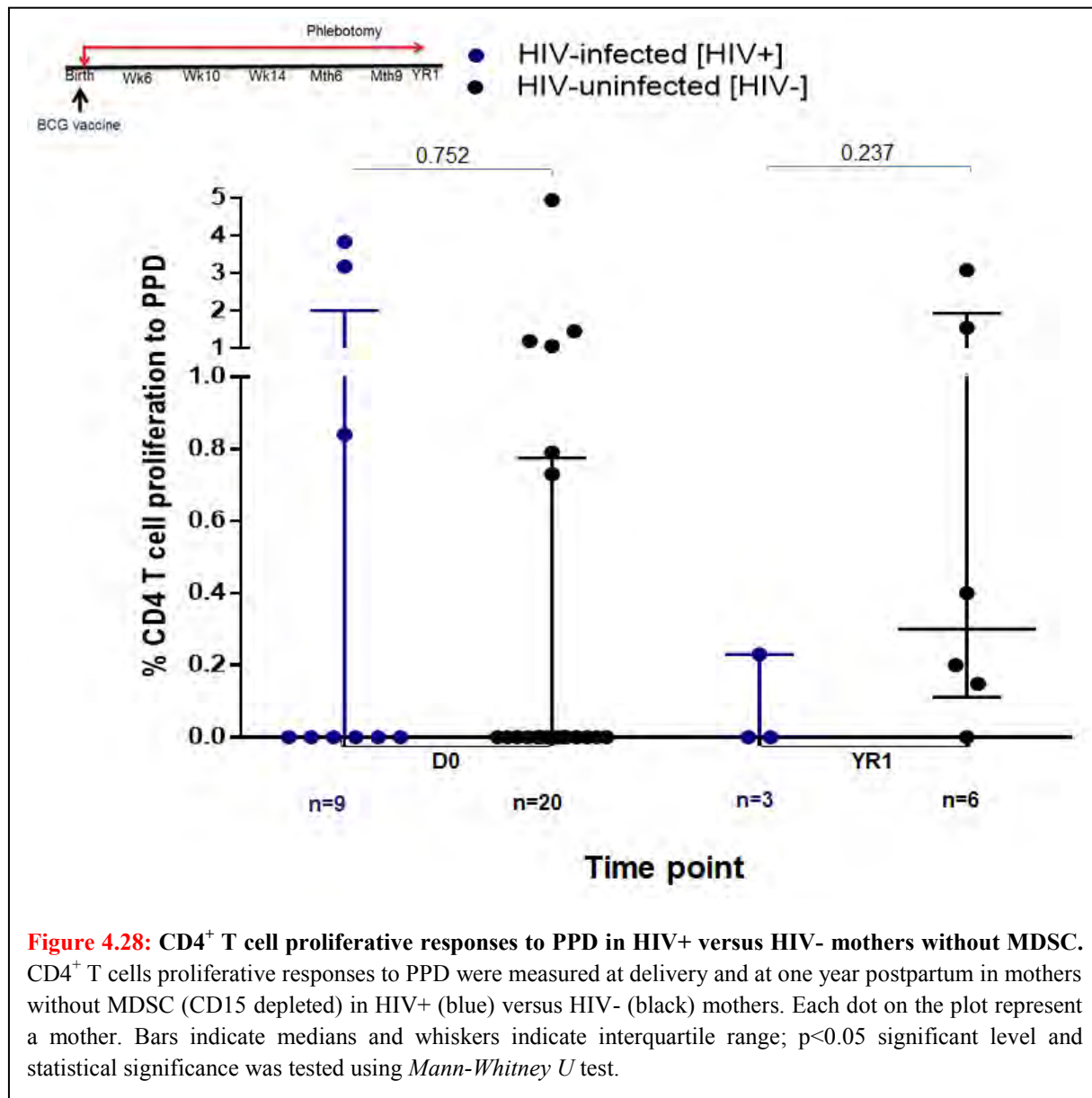
4.3.2.17 PPD induced CD4⁺ T cell proliferation in HIV+ versus HIV- mothers

We looked at the frequency of proliferating T cells to PPD between HIV+ versus HIV- mothers at delivery and at one year postpartum. As shown in [Figure 4.27](#), there were no significant differences in CD4⁺ T cell proliferative responses between groups at either time point.



4.3.2.18 PPD induced CD4⁺ T cell proliferation in HIV+ versus HIV- mothers after MDSC depletion

We also evaluated if depleting MDSC would have different effects in HIV infected versus uninfected mothers on CD4⁺ T cell proliferation to PPD at delivery and at one year postpartum. As shown in Figure 4.28, there were no significant differences in CD4⁺ T cell proliferative responses between groups observed.



4.3.2.19 Dynabead induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in infants

Anti-CD3CD28 (Dynabead) was employed as a positive control in this study, as it has been shown to be strong T cell activator acting directly on T cell receptors and does not require feeder cells (antigen presenting cells) or antigens (Li *et al.*, 2010) (Oviedo-Orta *et al.*, 2010). To investigate the influence of MDSC on T cell proliferation to strong, non-specific stimulation, we evaluated CD4⁺ T cell proliferative responses to α -CD3CD28 (Dynabead) stimulation in whole PBMC and without MDSC in infants at birth, at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age [(Figure 4.29 (i)(a-c)]. Proliferation at all time points was robust. There were

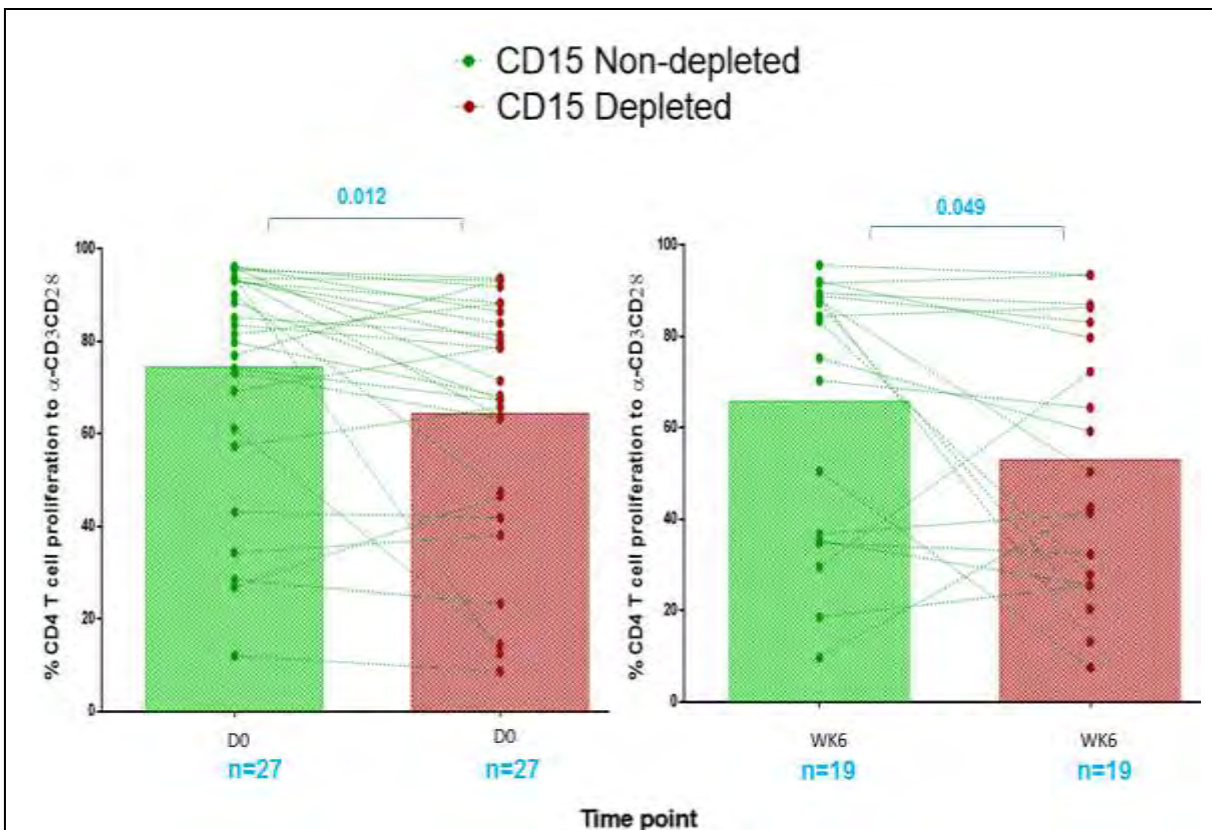


Figure 4.29 (i)(a): CD4⁺ T cell proliferative responses to Dynabead in infants in whole PBMC versus without MDSC in the individual time points. CD4⁺ T cells proliferative responses to Dynabead were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red). Matched pair of dots on the plot represent an infant. Bars indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using non-parametric *Wilcoxon matched-pairs* sign rank test*.

some significant differences observed, at birth the frequency of CD4⁺ T cell proliferative responses to Dynabead was significantly lower at birth (median of CD15 Non-depleted=83.600% versus CD15 Depleted=68.30% and $p=0.012$) and at 6 weeks of age (median of CD15 Non-depleted=62.000% versus CD15 Depleted=54.700% and $p=0.049$) in infants after MDSC depletion but not at 10 and 14 weeks ($p=0.548$ and $p=0.074$ respectively); or at 6 and 9 months ($p=0.425$ and $p=0.168$ respectively) and at one year ($p=0.998$) [(Figure 4.29 (i)(a-c)]. Also, a significantly lower T cell proliferation to Dynabeads was observed in the grouped time point analyses at birth and 6 weeks grouped time points after MDSC depletion (median of CD15 Non-depleted=84.160% versus CD15 Depleted=67.85% and $p=0.009$) [(Figure 4.29 (ii)]. In general, our observation shows that in response to Dynabead stimulation, proliferation decreased post MDSC depletion in infants.

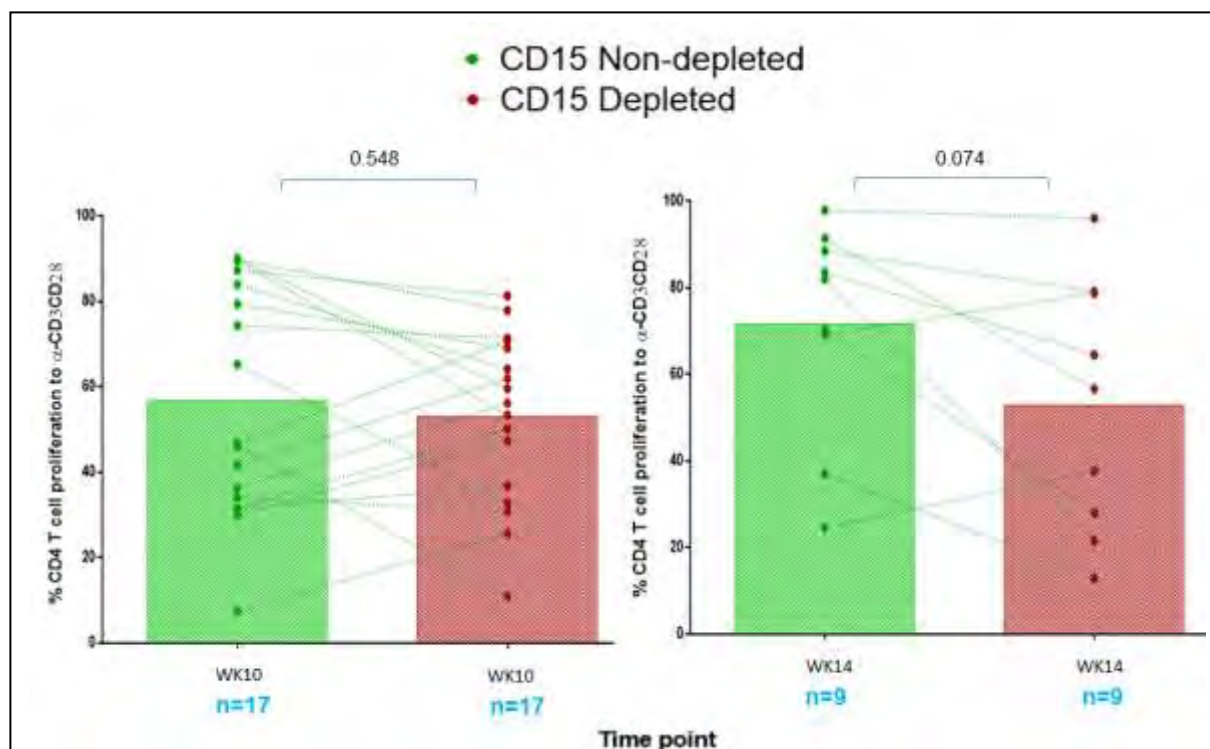


Figure 4.29 (i)(b): CD4⁺ T cell proliferative responses to Dynabead in infants in whole PBMC versus without MDSC in the individual time points. CD4⁺ T cells proliferative responses to Dynabead were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red). Matched pair of dots on the plot represent an infant. Bars indicate interquartile range; $p<0.05$ significant level and statistical significance was tested using non-parametric *Wilcoxon matched-pairs* sign rank* test.

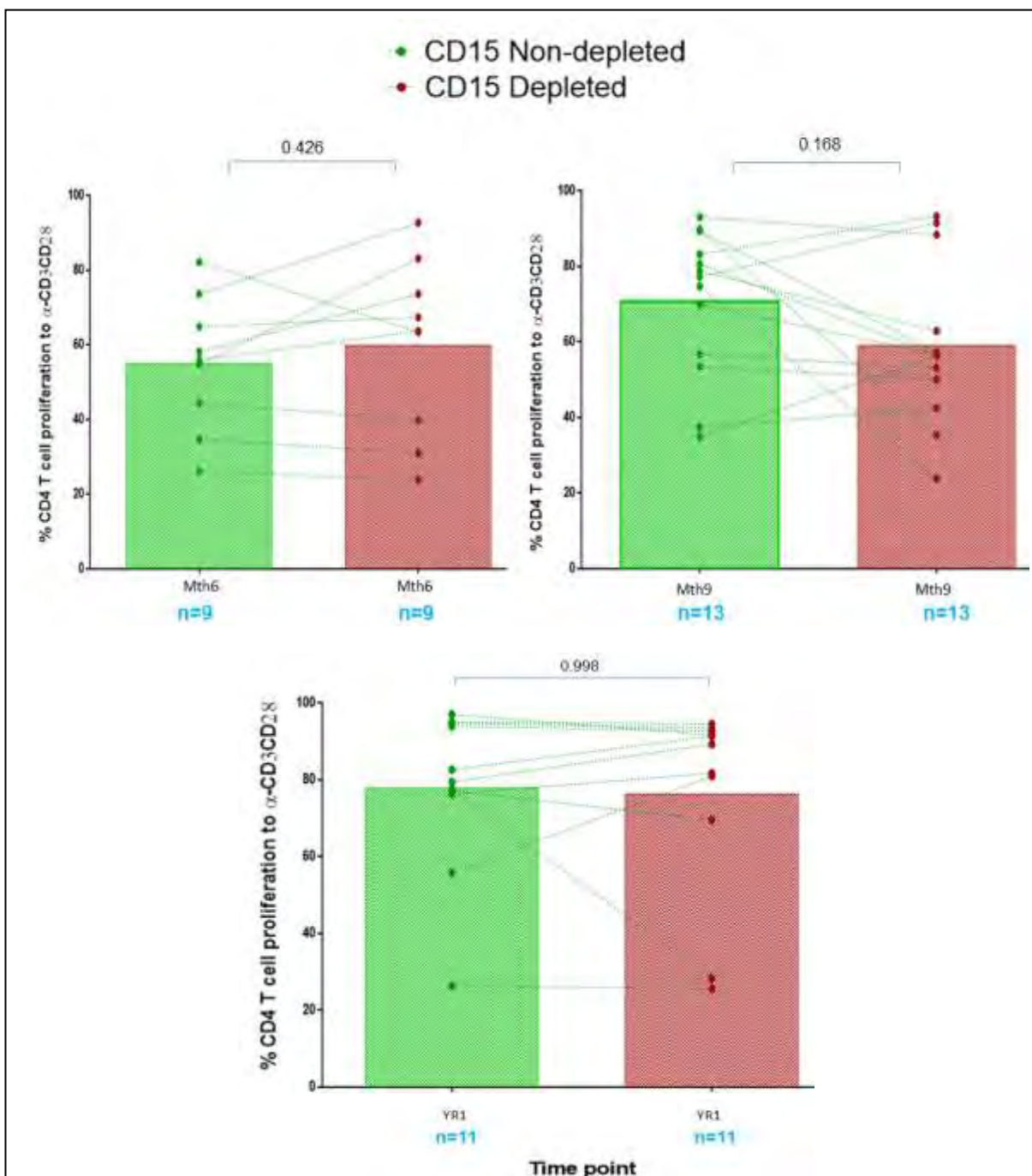


Figure 4.29 (i)(c): CD4⁺ T cell proliferative responses to Dynabead in infants in whole PBMC versus without MDSC in the individual time points. CD4⁺ T cells proliferative responses to Dynabead were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red). Matched pair of dots on the plot represent an infant. Bars indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using non-parametric *Wilcoxon matched-pairs* sign rank test*.

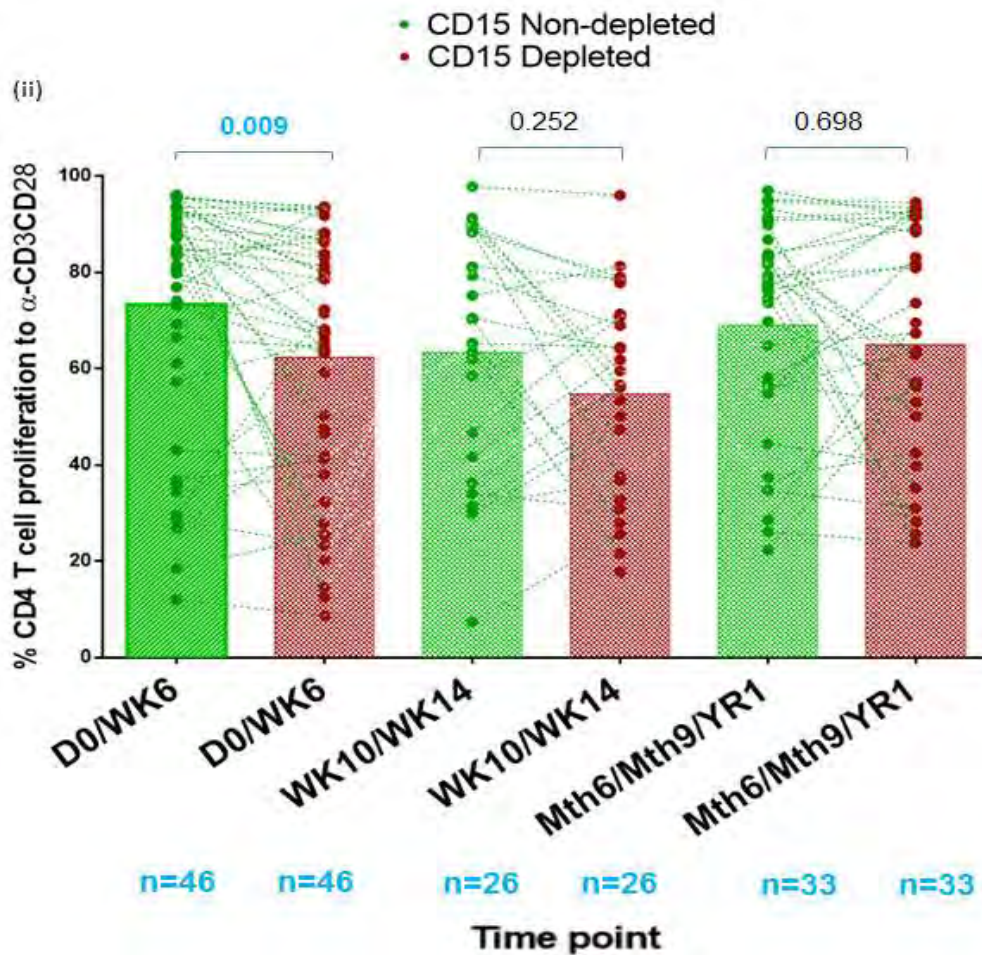


Figure 4.29 (ii): CD4⁺ T cell proliferative responses to Dynabead in infants in whole PBMC versus without MDSC in the grouped time points. In the grouped time point analyses, Dynabead responses were measured at birth and 6 weeks, at 10 and 14 weeks, and at 6 and 9 months and one year of age grouped time points. Matched pair of dots on the plot represent an infant. Bars indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using non-parametric *Wilcoxon matched-pairs* sign rank* test.

4.3.2.20 Dynabead induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in mothers

In mothers, the CD4⁺ T cell proliferative responses to Dynabead were evaluated in whole PBMC and without MDSC at delivery and at one year postpartum. As shown in Figure 4.30, there was a significantly higher CD4⁺ T cell proliferation in mothers at one year postpartum after MDSC depletion (median of CD15 non-depleted=79.650% versus CD15 depleted=88.060%; $p=0.016$), but not at delivery (median of CD15 non-depleted=72.910% versus CD15 depleted=60.110%; $p=0.686$).

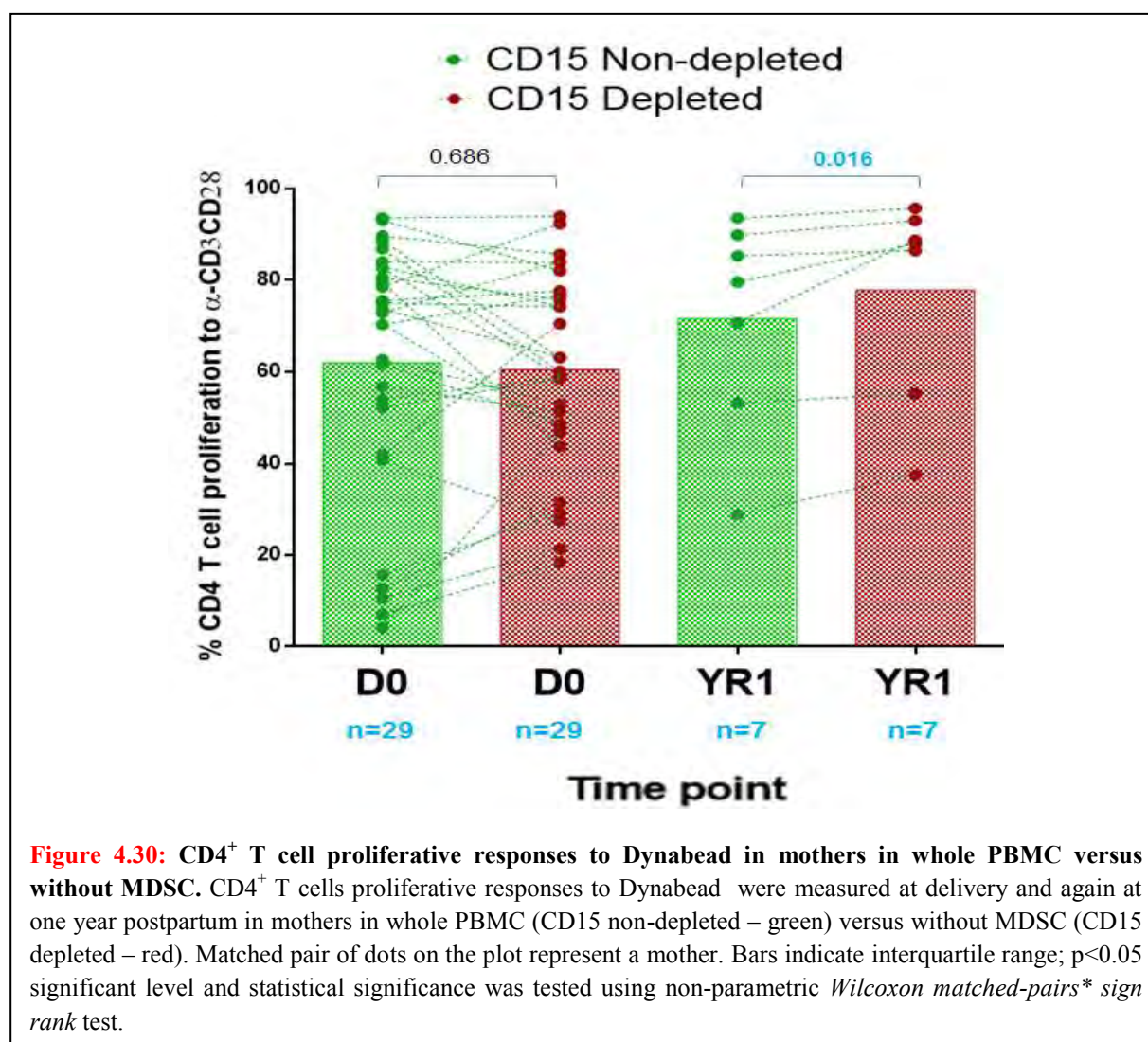
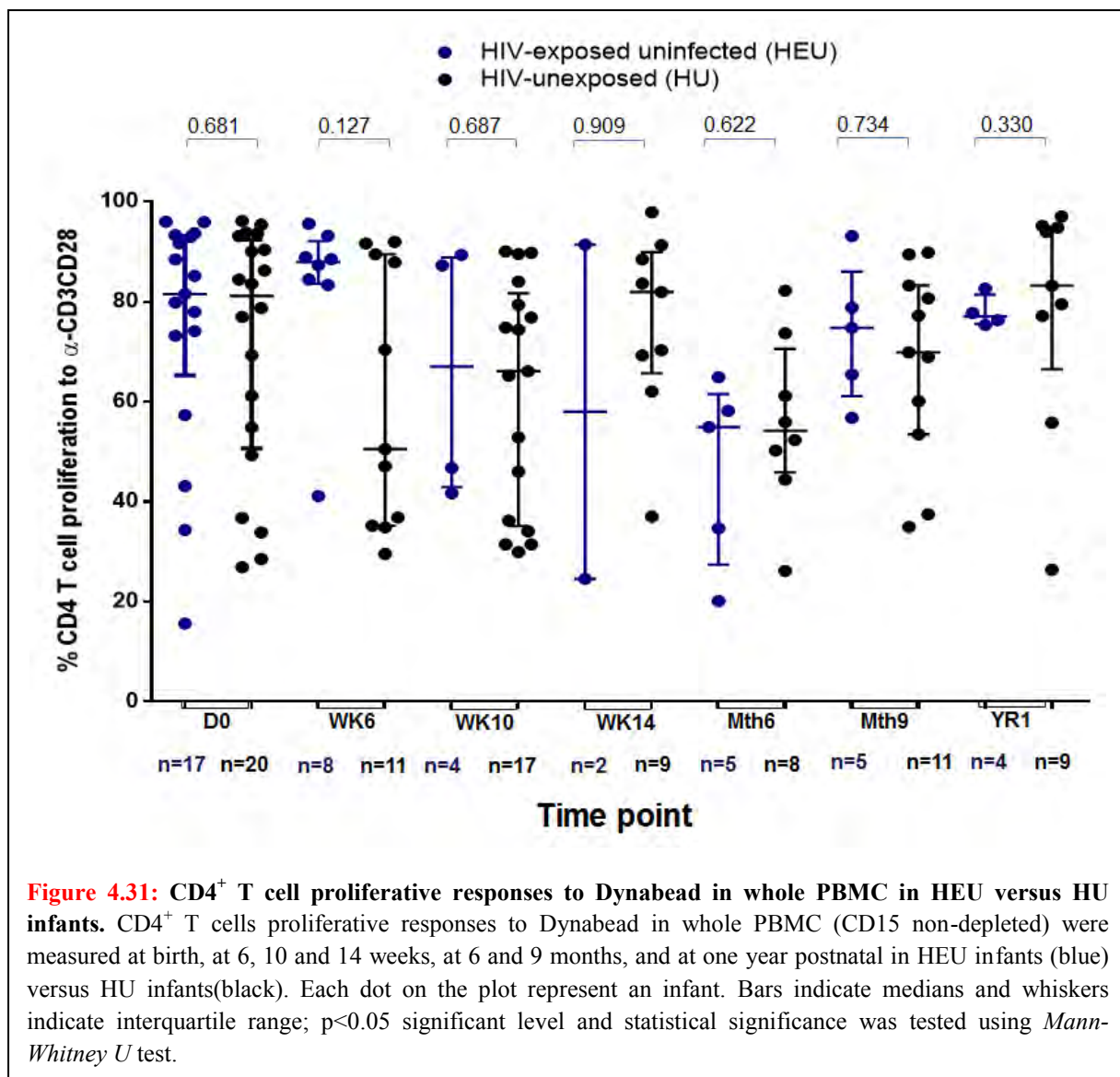


Figure 4.30: CD4⁺ T cell proliferative responses to Dynabead in mothers in whole PBMC versus without MDSC. CD4⁺ T cells proliferative responses to Dynabead were measured at delivery and again at one year postpartum in mothers in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red). Matched pair of dots on the plot represent a mother. Bars indicate interquartile range; $p<0.05$ significant level and statistical significance was tested using non-parametric *Wilcoxon matched-pairs* sign rank* test.

4.3.2.21 Dynabead induced CD4⁺ T cell proliferation in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants

To evaluate if HIV-exposure would influence CD4⁺ T cell proliferation to strong, non-specific stimulation, we looked at the frequency of proliferating T cells to Dynabead stimulation in whole PBMC between HEU versus HU infants at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age. As shown in [Figure 4.31](#), there were no significant differences in CD4⁺ T cell proliferative responses between groups.



4.3.2.22 Dynabead induced CD4⁺ T cell proliferation after MDSC depletion in HEU versus HU infants

We evaluated the frequency of proliferating T cells to Dynabead stimulation in the absence of MDSC between HEU versus HU infants at birth; 6, 10 and 14 weeks; at 6 and 9 months and at one year of age. As shown in **Figure 4.32**, there were no significant differences in CD4⁺ T cell proliferative responses. T cell proliferation was robust in both HEU and HU infants after Dynabead stimulation.

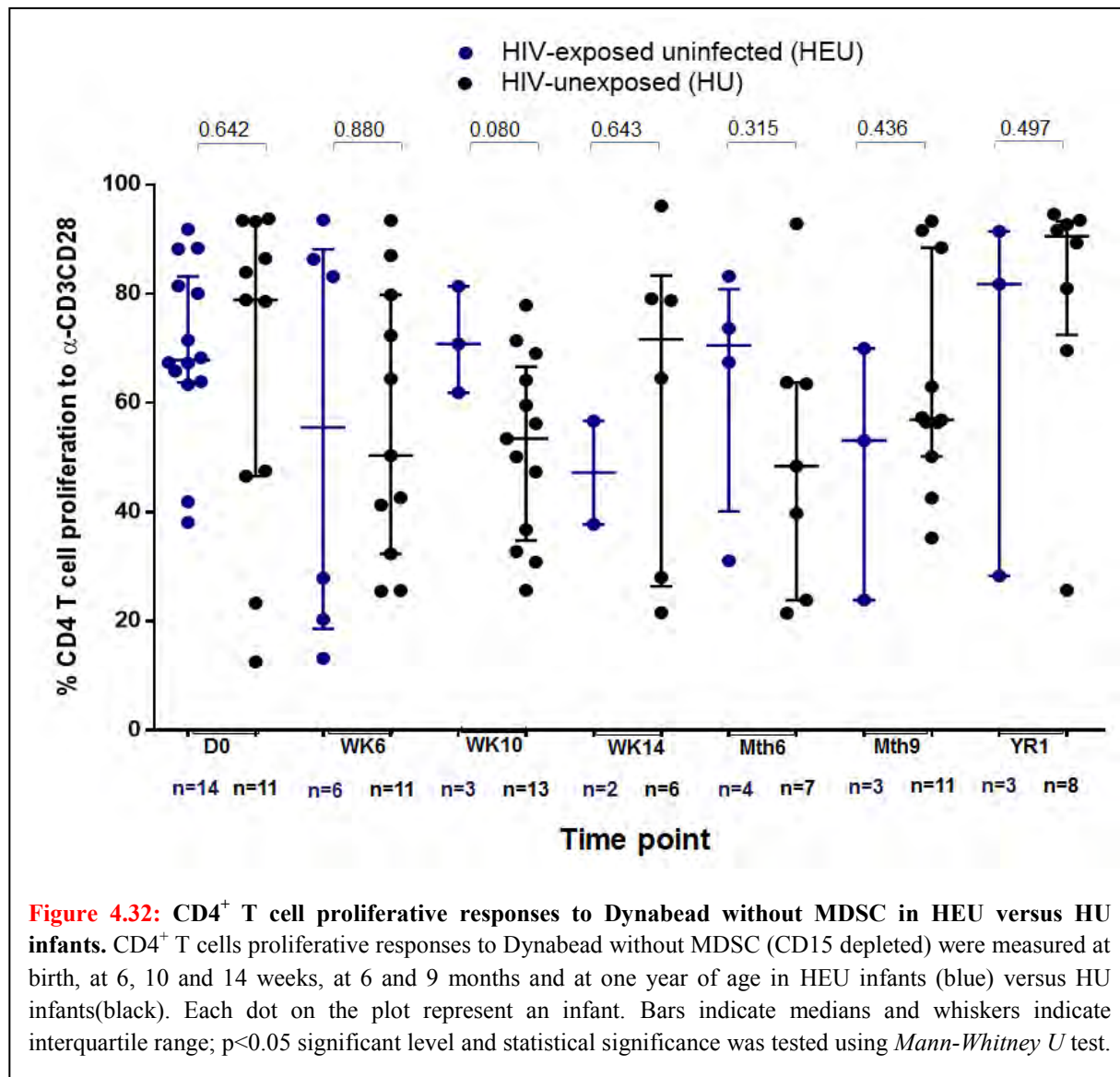
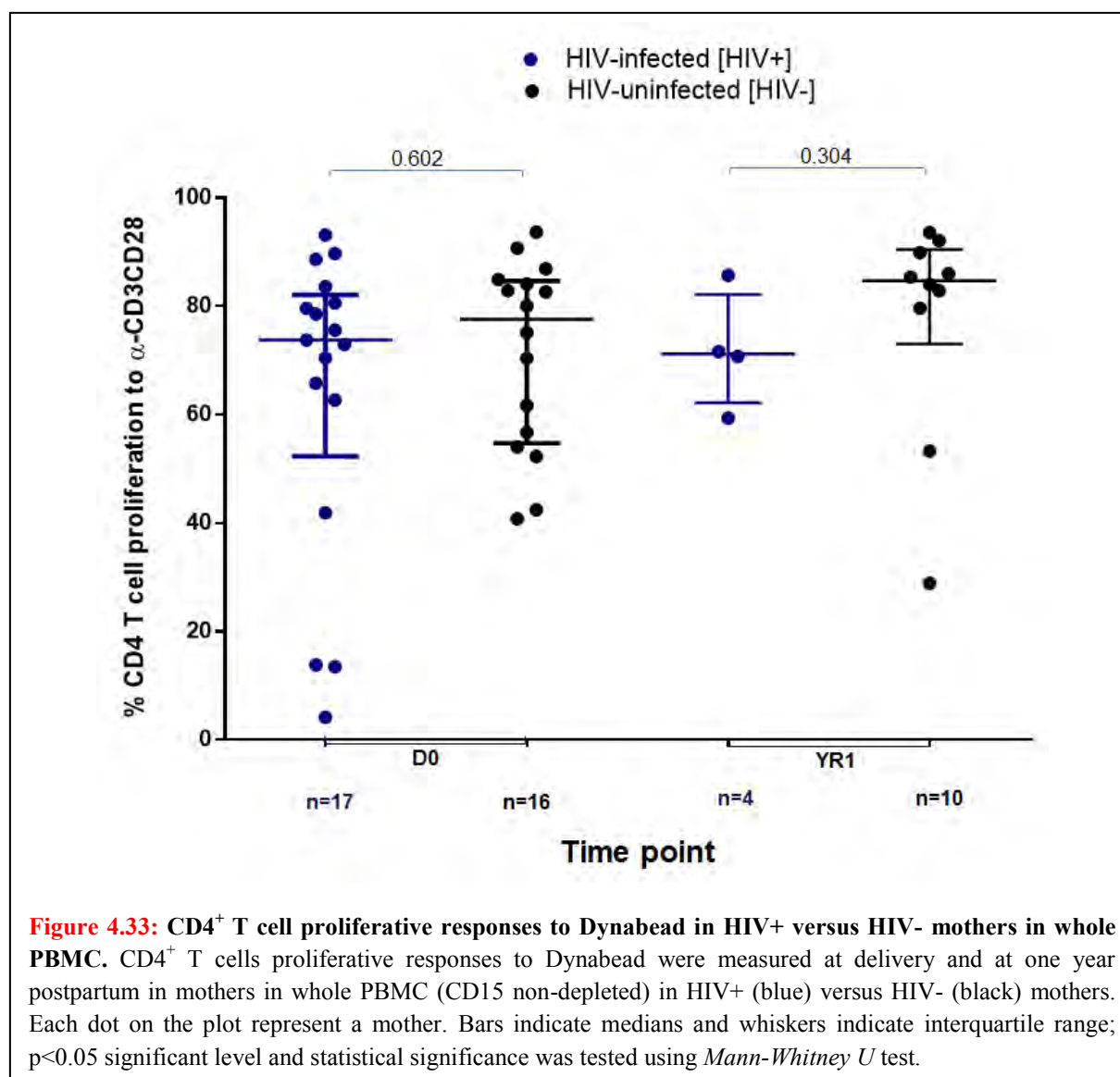


Figure 4.32: CD4⁺ T cell proliferative responses to Dynabead without MDSC in HEU versus HU infants. CD4⁺ T cells proliferative responses to Dynabead without MDSC (CD15 depleted) were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in HEU infants (blue) versus HU infants (black). Each dot on the plot represents an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

4.3.2.23 Dynabead induced CD4⁺ T cell proliferation in HIV+ versus HIV- mothers

We evaluated if HIV infection in mothers would affect CD4⁺ T cell proliferation to strong, non-specific stimulation. We investigated the frequency of proliferative T cells to Dynabead in the presence of MDSC (CD15 Non-depleted) between HIV+ versus HIV- mothers at delivery and at one year postpartum. As shown in **Figure 4.33**, there were no significant differences observed in CD4⁺ T cell proliferative responses between groups at all time points.



4.3.2.24 Dynabead induced CD4⁺ T cell proliferation in HIV+ versus HIV- mothers after MDSC depletion

We evaluated if MDSC depletion would influence CD4⁺ T cell proliferation to strong, non-specific stimulation in HIV infection. We looked at the frequency of proliferating T cells to Dynabeads in the absence of MDSC (CD15 Depleted) between HIV+ versus HIV- mothers at delivery and at one year postpartum. There were no significant differences in CD4⁺ T cell proliferative responses between groups at all time points. However, at 1 year postpartum the number of HIV+ mothers for which Dynabead proliferative responses were measured was small and statistical analysis could not be performed; data not shown).

Table 4.6: Summary of the results of all infant and mothers CD4⁺ T cell proliferation to vaccines and antigens in PBMC (presence of MDSC) versus without MDSC (after MDSC depletion) in infants and mothers. P-values of infants and mothers CD4⁺ T cell proliferation in PBMC (presence of MDSC) versus without MDSC (after MDSC depletion) after stimulation with the following antigens [Hepatitis B (Hep B), Tetanus toxoid (TT), Purified Protein Derivatives of *M. bovis* (PPD) and anti-CD3CD28 (Dynabead)]. Blue highlights represent the parameters (p-values and type of data sets), Red highlights represent the different time points [birth (D0), 6, 10 and 14 weeks (WK6, WK10 and WK14), 6 and 9 months (Mth6 and Mth9), and 1 year (YR1)], and Purple highlights represent significant differences in CD4⁺ T cell proliferation in the presence of MDSC versus after MDSC depletion to vaccine antigens. GRP=Group, Indv=Individual, TP= Time point, B=Baby, and M=Mother.

Parameters	p-values						
Time points	D0	WK6	WK10	WK14	Mth6	Mth9	YR1
Hep B Indv. TP (B)			0.125	0.998	0.094	0.625	0.031
Hep B GRP TP (B)			0.769		0.209		
Hep B Indv. TP (M)	<0.001						0.063
TT Indv. TP (B)			0.158	0.125	0.750	0.438	0.501
TT GRP TP (B)			0.078		0.850		
TT Indv. TP (M)	0.012						0.250
PPD Indv TP (B)		0.375	0.813	0.313	0.998	0.383	0.779
PPD GRP TP (B)		0.998			0.775		
PPD Indv. TP (M)	0.532						0.438
Dynabead Indv TP (B)	0.012	0.049	0.548	0.074	0.426	0.168	0.998
Dynabead GRP TP (B)	0.009		0.252		0.698		
Dynabead Indv. TP (M)	0.686						0.016

Table 4.7: Summary of the results of all infant and mothers CD4⁺ T cell proliferation to vaccines and antigens in HEU versus HU infants as well as in HIV+ versus HIV- mothers in PBMC (presence of MDSC) and without MDSC (after MDSC depletion). P-values of infants and mothers CD4⁺ T cell proliferation in PBMC (presence of MDSC) and without MDSC (after MDSC depletion) in HEU versus HU infants as well as in HIV+ versus HIV- mothers after stimulation with the following antigens [Hepatitis B (Hep B), Tetanus toxoid (TT), Purified Protein Derivatives of *M. bovis* (PPD) and anti-CD3CD28 (Dynabead)]. Blue highlights represent the parameters (p-values and type of data sets), Red highlights represent the different time points [birth (D0), 6, 10 and 14 weeks (WK6, WK10 and WK14), 6 and 9 months (Mth6 and Mth9), and 1 year (YR1)], and Purple highlights represent significant differences in CD4⁺ T cell proliferation to vaccine antigens in the presence and absence of MDSC in HEU versus HU infants. GRP=Group, Indv=Individual, TP= Time point, B=Baby, M=Mother, N=MDSC non-depleted, and D=MDSC depleted.

Parameters	p-values						
Time points	D0	WK6	WK10	WK14	Mth6	Mth9	YR1
Hep B GRP TP (B) (N)			0.232		0.613		
Hep B GRP TP (B) (D)			0.524		0.739		
Hep B Indv TP (M) (N)	0.626						0.775
TT Indv TP (B) (N)			0.796	0.723	1.000	0.493	0.672
TT GRP TP (B) (N)			0.914		0.391		
TT Indv TP (B) (D)			1.000	0.745	1.000	0.466	0.728
TT GRP TP (B) (D)			0.984		0.449		
TT Indv TP (M) (N)	0.981						0.443
PPD Indv TP (B) (N)		0.491	0.513	0.556	0.845	0.782	0.576
PPD GRP TP (B) (N)		0.636			0.905		
PPD GRP TP (B) (D)		0.277			0.517		
PPD Indv TP (M) (N)	0.910						0.117
PPD Indv TP (M) (D)	0.752						0.237
Dynabead Indv TP (B) (N)	0.681	0.127	0.687	0.909	0.622	0.734	0.330
Dynabead Indv TP (B) (D)	0.642	0.880	0.080	0.643	0.315	0.436	0.497
Dynabead Indv TP (M) (N)	0.602						0.304

4.4 Discussion

As much as characterising and evaluating the quantity and ontogeny of myeloid derived suppressor cells (MDSC) is important, evaluating their effect on vaccine responses is vital. T cell proliferation is an essential evaluation of vaccine immunogenicity and T cell function. MDSC are known to influence T cell proliferation to non-specific antigens. Rieber *et al.* showed suppression of T cell proliferation to α -CD3 supplemented with IL-2 by cord blood MDSC(Rieber *et al.*, 2013). Also, in response to α -CD3CD28 (Dynabead), we have shown that in neonates, MDSC dampens T cell responses in cord blood(Gervassi *et al.*, 2014). Little is known about the effect of MDSC on T cell proliferative responses to vaccines in infants (Gantt *et al.*, 2014)(Gervassi *et al.*, 2014).

In this chapter, we evaluated CD4⁺ T cell proliferation in response to Hep B and TT antigens, and to *Mycobacterium bovis* tuberculin PPD and Dynabead antigens. Generally, there was an overall good T cell proliferation to Hep B vaccination, but very low T cell proliferation to TT in infants post vaccination and in mothers at all time points. T cell proliferation to PPD was good post BCG vaccination in infants. As a caveat of the study, understanding that that PPD is a cell-free purified protein fraction obtained from a human strain of *Mycobacterium tuberculosis*(Willett, 2014), we cannot completely rule out the impact of environmental *Mycobacteria tuberculosis* exposure when measuring PPD responses. However, the influence of environmental *M. tb* has been shown to be minimal(Mandalakas *et al.*, 2015)(Hesseling *et al.*, 2015). Moreover, there were some infants who responded poorly to all the vaccines assessed.

There were little and in most cases no significant differences at most grouped time point analyses in MDSC frequency and absolute count between infants whose CD4⁺ T cell responded and those who had poor CD4⁺ T cell proliferation to most vaccine antigens. Also, maternal MDSC frequency was not significantly different in responders versus non-responders to all the vaccines stimulation. Moreover, we hypothesized that MDSC frequency will influence CD4⁺ T cell proliferation negatively, but contrary to our hypothesis in infants, there was more often a trend towards high MDSC frequency and counts in responders compared to

non-responders to most vaccine antigens. This may have been because we compared the *in-vitro* response to these antigens to the MDSC count and frequency at the same time point. MDSC may influence vaccine responses *in-vivo* at the time the vaccine is given, rather than influence the longevity of the later responses. Furthermore, since MDSC were present at higher frequencies at birth, and waned at later time points, their effects in older infants may be minimal.

In chapter 3, we showed that granulocytic MDSC are elevated at birth and rapidly decrease from 6 weeks onwards, this may account for the lack of effect of MDSC depletion on T cell proliferation to vaccines. Also, as one of the limitations of the study, MDSC were depleted by CD15 antibody selection for G-MDSC, thus, the suppressive effect of M-MDSC (though less than 1% present in healthy infants and adults) cannot be underestimated (Gervassi *et al.*, 2014). The study is still accruing data, and with larger numbers, an effect on vaccine T cell proliferative responses may become apparent. Alternatively, MDSC may not have an influence on proliferative responses to vaccine antigens, or the effect of other suppressor cells elevated during gestation may alter the effect of MDSC. Also, MDSC may be vaccine and antigen dependent, playing a different role on the immune system yet to be understood.

Possible other reasons for the lack of MDSC effect on T cell proliferation to vaccine antigens could be that the three to four days incubation period employed for cell culture was too long, and MDSC are short-lived cells; but cells were assessed for viability and 0.02% of 0.5ug/mL recombinant human IL-7 (rhIL-7) was added daily during incubation. In future, as a recommendation, studies using the CFSE incorporation assay should perhaps evaluate the effect of culturing cells in a fewer number of days (one to two days). Also, we did not evaluate CD8⁺ T cell proliferative responses; we might be ignoring responses of cytotoxic T cell proliferation. In the mothers, the effect of MDSC to vaccine antigens was limited.

Although all mothers will have received BCG and TT vaccination in infancy, only mothers younger than 22 years of age will have received Hep B. A large proportion of mothers will have been exposed to M. TB and environmental mycobacteria. Also, most of the mothers would probably be exposed to Hep B infection and only a proportion of the mothers are likely to have

been cleared of the Hep B infection. Hep B may be an innate ligand in the unvaccinated mothers. Furthermore, the prevalence of Hepatitis B is approximately 80% in women of childbearing age in South Africa (Matthews *et al.*, 2015) (Schweitzer *et al.*, 2015). Therefore, responses in mothers may not be measuring vaccine immunogenicity, but rather response to natural infection.

We found that MDSC may dampen the *in-vitro* recall response to some antigens, particularly, Tetanus and Hepatitis B. In the matched analyses there were significantly higher proliferative responses observed to these antigen stimulations after the depletion of CD15⁺ MDSC. In infants, at one year of age (post the last dose of Hep B vaccination and last follow-up time point) after Hep B stimulation in the individual time point's analyses, there was a significantly higher T cell proliferation after MDSC were depleted compared to whole PBMC. In the mothers, significant differences were evident; we found significantly higher CD4⁺ T cell proliferative responses to both Hep B and TT vaccine stimulation after MDSC depletion compared to whole PBMC immediately postpartum. To the best of our knowledge, this study is the first to evaluate the effect of MDSC on T cell proliferation to vaccines in infants; and also, the first study to evaluate the effect of MDSC on T cell proliferative responses in mothers. The non-responders were both HEU as well as HU infants, and HIV⁺ and HIV⁻ mothers was not mainly HEU infants or HIV⁺ mothers.

After Dynabead stimulation, there were some significant differences observed. In infants, contrary to our hypothesis, at birth and at 6 weeks of age there were significantly lower CD4⁺ T cell proliferative responses after MDSC depletion compared to whole PBMC and a trend at 14 weeks, and this was evident in the grouped time point analyses at birth and 6 weeks grouped time points. This is contrary to our finding in Gervassi *et al.* where we showed that MDSC dampens T cell proliferative responses in infants post Dynabead stimulation (Gervassi *et al.*, 2014). The possible reason for this may be due to geographical, genetic, exposure to other pathogens and mode of delivery as infants in both studies were residing in different countries. Also, at the early time points at birth and 14 weeks since the MDSC frequency is expected to be more, the effect of depletion could probably have been felt more than at the later time points when MDSC frequency is expected to be low. However, this finding would warrant further investigation.

Also, at other time points there were trends towards lower T cell proliferation after MDSC depletion at 6 weeks and at 9 months of age post Dynabead stimulation. In mothers, in conformity with our hypothesis, after Dynabead stimulation at one year postpartum there was a significantly higher CD4⁺ T cell proliferation after MDSC depletion versus in whole PBMC. The increase in CD4⁺ T cell proliferation at one year post MDSC depletion but not at delivery could possibly be due to the fact that the frequency of MDSC at a one year postpartum as shown in [Chapter 3](#) would be lower compared to immediately after gestation. Even though MDSC was depleted at delivery, the effect could still be felt due to the high concentration of MDSC at delivery.

Our results contrast our findings in Gervassi *et al.* where we showed that MDSC suppressed *in-vitro* cord blood T cell responses to Dynabeads([Gervassi et al., 2014](#)). However, there were some differences between this study and that of Gervassi *et al.* The infants in this study were recruited from normal vaginal delivery exposed to maternal vaginal microbiome and some exposed to *in-utero* HIV-infection which may play a vital role in the developing infant immunity([Mueller et al., 2014](#))([Valdez et al., 2014](#)), while the infants in the Gervassi *et al.* study were born from caesarean-sections and non-exposed to *in-utero* HIV-infection([Gervassi et al., 2014](#)). All of theirs were American babies in tertiary care settings, ours were all Africans. Exposure to helminths prevalent in sub-Saharan Africa, tuberculosis and other infections *in-utero* have been shown to be associated with high MDSC frequencies([Van Ginderachter et al., 2010](#))([du Plessis et al., 2013](#)).

In previous studies, we found significantly higher proliferative responses to vaccine antigens and non-specific stimuli in HEU versus HU ([Kidzeru et al., 2014](#)). These differences were not seen here. This could be due to differences between the two studies. In the previous study we evaluated responses in a larger number of HEU infants (n=46) versus a similar sample size of HU infants. Also, we employed the whole blood assay as well as Ki67 proliferation assay while in this study the CFSE proliferation assay was used. HIV-exposure did not alter T cell proliferation in infants.

We hypothesised that HIV-exposure and HIV infection may contribute to the effect of MDSC on T cell proliferation to vaccines. However, we found no such influence. This could be secondary to small numbers or possibly due to a true lack of difference in MDSC frequency and function in HIV-exposed infants. As much as HIV-exposure did not affect T cell proliferation before and after MDSC depletion, HIV infection did not in response to all vaccine and antigen stimulation in mothers, and this was observed both at delivery and at one year postpartum. However, there was a trend towards higher T cell proliferative responses in HIV negative mothers in response to Hep B in whole PBMC and after MDSC depletion. We cannot rule out the option that it could be as a result of past infection, since Hep B is known to upregulate MDSC(Pallett *et al.*, 2015)(Weber, 2015).

Generally, mothers had very low T cell proliferative responses to TT, this shows that, it would be advantageous for mothers to be given boosters if cellular immune responses to TT are important. It is unlikely that mothers had been vaccinated as in South Africa pregnant women are not routinely given Tetanus toxoid vaccine boosters during gestation.

To the best of our knowledge, this study is the first to evaluate the effect of MDSC on T cell proliferation to vaccines in infants; and also, the first study to evaluate the effect of MDSC on T cell proliferative responses in mothers. However, earlier studies have assessed the effect of MDSC on T cell proliferation in adults. The significant increase in T cell proliferation in mothers after MDSC depletion versus in whole PBMC at delivery in line with our hypothesis, could be supported as a result of several factors; The immune system of pregnant women is compromised and largely suppressed to accommodate for optimum viviparity, with high frequency of Tregs and other suppressive mechanisms including MDSC(Burt, 2013)(Gantt *et al.*, 2014)(Bansal, 2010)(Nair *et al.*, 2015)(Ostrand-rosenberg *et al.*, 2012). The increased frequency of regulatory cells may contain a high proportion of MDSC to create a state of equilibrium in the maternal immune system. The lack of significant difference at one year postpartum may be due to the fact that the quantity of MDSC fades out after gestation to level that would have little effect on T cell proliferation as expected.

The study as described has a few limitations; retention of participants in the study was challenging. Depletion was not always 100% and there was not always enough cells to do the assays, due to difficulty in collecting large volumes of blood from infants. The need for 10^6 cells / well to perform the CFSE proliferation assay which at most times points was a major challenge collecting whole blood samples from infants. For the CD15 depletion assay, the lack of difference may be due to a function of the assay. After depletion using EasySep[®] magnetic cell separation technique, cells may be less “happy” compared to cells that have not gone through the same process. However, experiments where CD15 enriched cells are added back to CD15 depleted cell are in progress and should may be provide a better comparison between MDSC presence and absence, since all cells would have gone through the same process. Furthermore, most responses were enhanced after CD15 depletion.

4.5 References

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CHAPTER 5

CYTOKINE PRODUCTION TO VACCINES AND ANTIGENS IN WHOLE PBMC AND WITHOUT MYELOID DERIVED SUPPRESSOR CELLS (MDSC) FROM BIRTH TO ONE YEAR POSTPARTUM

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5.1 Introduction

Neonates have limited capacity to produce IFN- γ and therefore, impaired cell-mediated immune responses against intracellular pathogens(Siegrist, 2007)(Ota et al., 2012). BCG vaccination at birth significantly enhances Th1 responses to mycobacterial antigens(Burl et al., 2012). There is evidence that IFN- γ production increases with age in infants(Mazzola, Marcos, & Abramczuk, 2011).

Several studies have shown that MDSC can suppress cytokine production, including IL-2 by CD4⁺ T cells and IL-13, IL-6 and G-MCSF in plasma to SIV vaccination in macaques(Sui et al., 2014). Also, reduced TNF- α , IL-10 and IFN- γ production by CD4⁺ T cells to anti-CD3CD28, and G-CSF, G-MCSF and MIP-1 β in plasma(du Plessis et al., 2013). The immune suppressive effect of MDSC on cytokine responses have been widely described in non-human primates(Guo et al., 2012)(Sui et al., 2014) and humans with cancer(Raychaudhuri et al., 2011). In patients with glioblastoma, accumulation of MDSC in peripheral blood has been shown to contribute to suppression of IFN- γ production by CD4⁺ T cell after stimulation with anti-CD3CD28(Raychaudhuri et al., 2011). However, in mice studies T cells producing IFN- γ have been shown to promote MDSC production in tumour-bearing mice(Guo et al., 2012).

There is limited data on the effect of MDSC on vaccine cytokine responses. Most of the studies have looked at response to non-specific stimulation. Raychaudhuri et al. showed that, MDSC suppress IFN- γ production in response to anti-CD3CD28 (Raychaudhuri et al., 2011). Also, it has been shown that IFN- γ may contribute to the increase of MDSC in mice exposed to cyclophosphamide(Guo et al., 2012). Rieber and colleagues showed that neonatal MDSC suppress Th1 (IFN- γ), Th2 (IL-5) and Th17 (IL-17) cytokine production by adult PBMC(Rieber et al., 2013).

As mentioned earlier, we and others have shown that the immune system of HIV infected and exposed infants is compromised and makes them susceptible to infections(Epalza et al., 2011), and alter their cytokine responses to vaccines(Miles et al., 2010)(Kidzeru et al., 2014)(Tchakoute et al., 2014). Qin et al. and others have showed that expansion of MDSC in HIV infection was associated with decreased IFN- γ production by T cells in HIV-1 positive

patients (Qin et al., 2013) (Gama et al., 2012). However, levels of induced MDSC were also associated with reduced protective immune responses to SIV in SIV vaccinated macaques (Sui et al., 2014).

Little is known about the effect of MDSC on cytokine production in infants and in mothers postpartum. Hence, we aimed to assess the effect of MDSC on cytokine response to vaccine antigens [Bacillus Calmette-Guérin (BCG), *Bordetella pertussis* (BP) for acellular pertussis (aP) vaccine, Tetanus toxoid (TT) and Hepatitis B surface antigen (HepB)]. This was performed by enumerating antigen specific IFN- γ producing cells using the enzyme-linked immunospot (ELISpot) assay in whole CBMC and/or PBMC, and MDSC depleted (CD15⁻) CBMC and/or PBMC cultures after stimulating with optimized concentrations of the respective antigens. We performed these on samples collected at birth (Day 0), followed-up at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age in infants. In mothers was at birth and again at one year postpartum.

5.2 Materials and methods

As described in [section 3.2.1](#), by using the standard ficoll gradients, cord blood mononuclear cells (CBMC) and peripheral blood mononuclear cells (PBMC) were isolated, and red blood cells (RBC) were depleted using glycophorin A micro beads. Further, as described in [section 4.2.1](#), after several calculations based on the number of cells required ([see Table 4.1](#)), cells were allocated for MDSC depletion (CD15⁻), as well as for the non-depleted experiments. Depletion of MDSC was performed by depleting CD15⁺ cells from whole CBMC and/or PBMC by employing the EasySep[®] magnetic cell separation technique.

5.2.1 IFN- γ enzyme-linked immunospot (ELISpot) assay

After obtaining all the population of cells required for ELISpot i.e. a portion with MDSC that have not had CD15⁺ cells depleted (whole CBMC and/or PBMC), and a portion without MDSC with CD15⁺ cells depleted (CD15⁻); the IFN- γ ELISpot assay was performed. The IFN- γ ELISpot assay is incubated for two days in 96 well flat bottom ELISpot polyvinylidene difluoride (PVDF) membrane plates (Millipore MultiScreen-IP Filter Plate).

5.2.1.1 IFN- γ enzyme-linked immunospot (ELISpot) assay [Day 1]

On the first day of the assay (Day 1), pre-labelled 96 well flat bottom ELISpot polyvinylidene difluoride (PVDF) membrane plates were coated with 100uL/well of 10ug/mL anti-human IFN- γ monoclonal primary antibody 1-D1K (MABTECH AB), in PBS, containing no protein [i.e. serum or bovine serum albumin (BSA)] ([Figure 5.1](#)). Any trace of protein may hinder the coating process and block the coating solution from penetrating the membrane of the wells ([Abcam, 1983](#)) ([ANIARA, 2008](#)) ([Brinkmann et al., 1993](#)). The plates were then covered with aluminium foil and incubated at 37⁰C with 5% CO₂ in a humidified incubator for ≥ 4 hours.

After incubation, the plates were removed from the incubator and excess coating solution washed off four times with 250uL/well of sterile PBS using a multichannel pipette. The wash steps were performed under aseptic conditions in a biosafety cabinet, and the membranes of the wells were prevented from drying out by disallowing the plate sit without liquid at any time. Using a multichannel pipette, 200uL of RPMI with 10% FCS was transferred to every washed well on

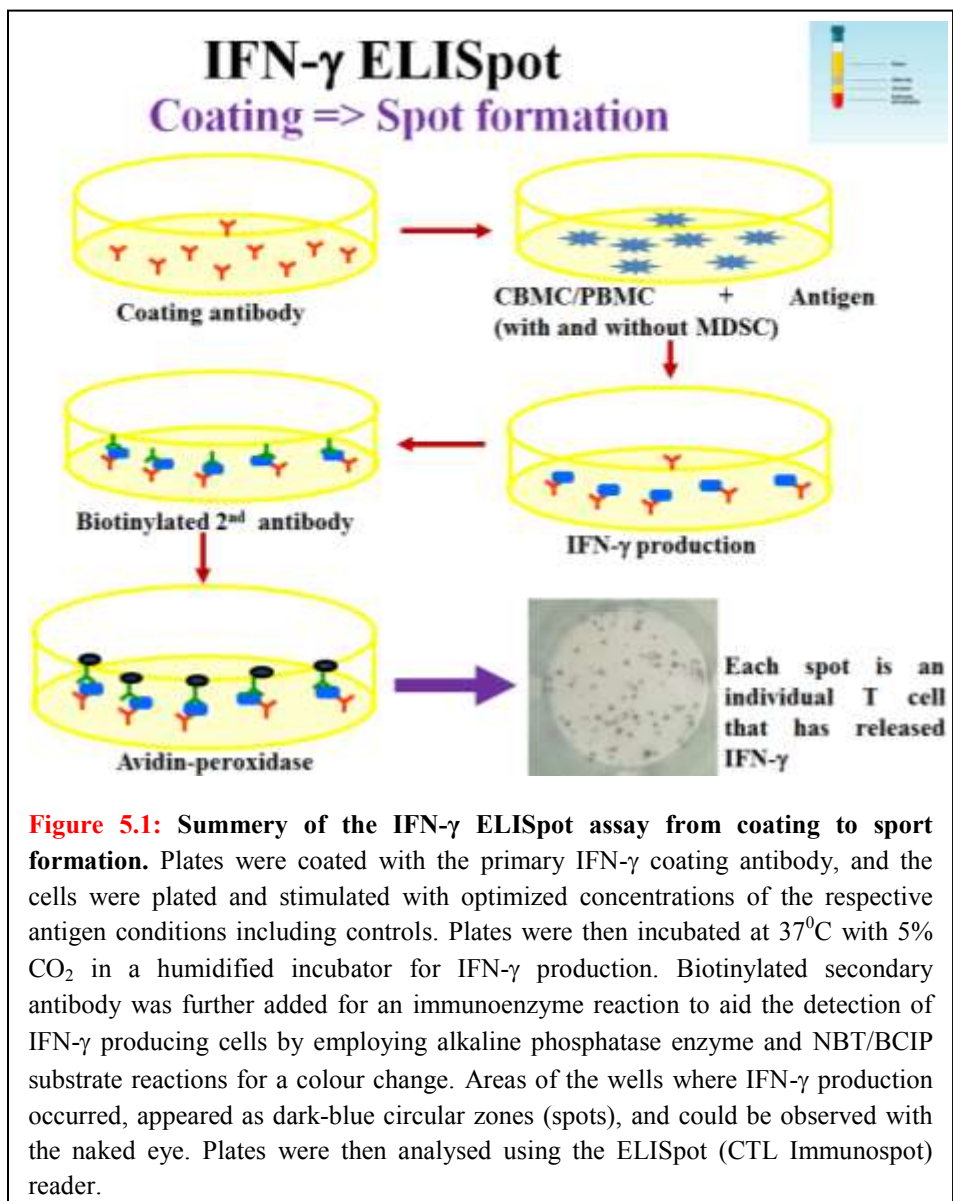
the plates (process known as blocking). The plates covered with aluminium foil were placed sterilely at 37°C with 5% CO₂ in a humidified incubator for ≥2 hours.

After, the plates were coated and blocked, approximately 2×10^5 cells/well (i.e. 2×10^5 cells/condition) of the different cell populations i.e. whole CBMC and/or PBMC with MDSC

and CD15⁺ without MDSC, were cultured for 18 – 22 hours in 100uL/well RPMI containing 1% penicillin-streptomycin

antibiotics and 15% Human Serum at 37°C with 5% CO₂ in a humidified incubator (Figure 5.1).

For each antigen, the cells were plated in duplicate wells (Figure 5.2). The cell culture was stimulated with optimized concentrations of the respective antigens



(25uL/well) to measure the number of IFN-γ producing cells in response to various vaccines and antigens in infants and their mothers. Specifically, the following antigens were added: 5×10^5

CFU/mL *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) [Danish strain 1331; SSI], 5ug/mL Tetanus toxoid (TT) [TETAVAX, Aventis Pharma (Pty) Ltd], 5ug/mL Pertussis antigens (BP) [Difco™ Bordetella Pertussis Antigen, BD] and 5ug/mL Hepatitis B surface antigen (Hep B) [Advanced Immunochemical Inc.]. Also, *in vitro* stimulation with 5ug/mL Phytohaemagglutinin (PHA) [PHA-P (Murex), Remel] as positive control, and cells alone with medium as negative control (CA), and a test control medium alone (Media) for internal quality control to monitor if there was any contamination in the incubation process. In addition to TT antigen, 0.5ug/mL BD Fastimmune™ CD28/CD49d co-stimulation was added.

Plates were placed in the incubator within 30 mins of stimulation. The plates were incubated at 37°C with 5% CO₂ in a sterile, humidified incubator for 18-22 hours.

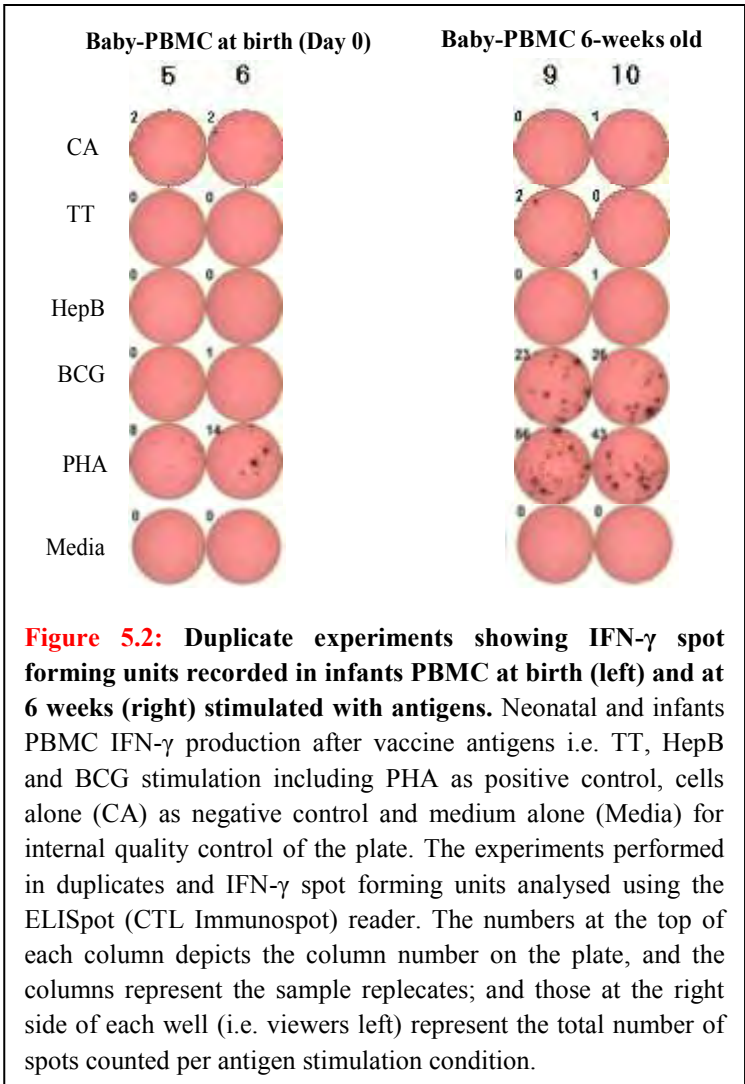
5.2.1.2 IFN- γ enzyme-linked immunospot (ELISpot) assay [Day 2]

On the second day of the assay (Day 2) following 18-22 hours of incubation, plates were removed from the incubator and supernatants collected. The plates were washed 4 times with 250uL/well of PBT/Tween-20 wash buffer. Bound human IFN- γ secreted by cells were demonstrated by means of an immunoenzyme [i.e. alkaline phosphatase-conjugated anti-biotinylated mouse anti-human IFN- γ monoclonal antibody (AP-anti-biotin)] procedure in which enzyme-substrate reactions were performed. To every well on the plate, the secondary antibody (AP-anti-biotin) was added, 100uL of 1ug/mL AP-anti-biotin (i.e. 10uL AP-anti-biotin in 10mL 0.5% BSA/PBS) was added and the plates incubated at RT for 2 – 4 hours (Figure 5.1).

Thereafter, the plates were washed 7 times with 250uL/well of PBT/Tween-20 wash buffer. Further, 100uL of 1.3ug/mL alkaline phosphatase-conjugated anti-biotin antibody [i.e. AP-anti-biotin antibody (enzyme)] (13.34uL AP-anti-biotin antibody in 10mL 0.5% BSA/PBS) was added to every well and the plate incubated at RT for 2-3 hours (Figure 5.1).

After incubation, the plates were washed 4 times with 250uL/well of PBT/Tween-20 wash buffer. Using a multichannel pipette with sterile pipette tips, 100uL of 1-Step™ nitro-blue tetrazolium and 5-bromo-4 chloro-3'-indolylphosphate (NBT/BCIP) for chromogenic blot (i.e. the substrates) from the reagent reservoir was transferred to each well of the plates and incubated

for 7 minutes at RT, or until distinct dark-blue circular zones (spots) appeared in the positive control wells (within manufacturer’s guideline of 5 – 15 minutes). The addition of NBT/BCIP to



the plates was done in a calculated fashion such that each well received the required 7 minutes incubation. The colour development in wells was stopped by rinsing each plate three times with 250uL/well of distilled water. The plates were kept inverted while drying overnight at RT.

The plates were analysed the following day (or within the next two working days) by using the ELISpot (CTL Immunospot) reader, allowing accurate estimation of the total amount of secreted IFN- γ /well.

Figure 5.2 shows a sample ELISpot plate generated in our lab. Comparisons were made between levels of IFN- γ obtained in the MDSC depleted and non-depleted

samples.

5.2.2 Data analysis

After ELISpot plates were analyzed using the ELISpot (CTL Immunospot) reader, the data was extracted into excel for further analysis.

5.2.2.1 Standard rules for cut-offs and controls for the ELISpot data

Following analysis by ELISpot (CTL Immunospot) reader, in order to prevent data bias and to perform background subtraction, the following rules were put in place:

1. Assay validity (before background subtraction):

The number of IFN- γ Spot Forming Units (SFU)/million cells in response to PHA was greater than the median plus 3 times the MAD of the negative control (i.e. Media) IFN- γ SFU/million cells, or the sample was excluded.

i.e. IFN- γ SFU/million cells [PHA] > IFN- γ SFU/million cells (Median + 3MAD)[Media]

[MAD=Median Absolute Deviation)

MAD is defined as the median of the absolute values of the deviation of each sample negative control number of spots from the negative control's median number of spots(Rouss *et al.*, 1993).

2. Background subtraction:

Number of IFN- γ SFU/million cells in response to the test antigens (i.e. BCG, BP, TT, HepB and PHA) minus background IFN- γ SFU/million cells in the media control)

i.e. (BCG, BP, TT, HepB and PHA induced IFN- γ SFU/million cells) – (IFN- γ SFU/million cells in the media).

This study was exploratory and obtaining whole blood samples from infants was challenging. From most infants, only a small volume of whole blood could be collected, so we define responses as the *magnitude of responses* measured(Alexander *et al.*, 2013)(Santos *et al.*, 2014).

However, in some studies in HIV infected adults and tuberculosis cases, ≥ 10 SFU/million cells after background subtraction have been used in ELISpot assays (Turk *et al.*, 2008) (Lawn *et al.*, 2007) (Aiken *et al.*, 2006).

Based on the literature, and on robustness of IFN- γ cytokine responses to the different antigens assessed, responses were considered positive (responsive) when IFN- γ cytokine producing cells to BCG were greater than or equal 20 SFU/million PBMC (≥ 20 SFU/ 10^6 PBMC), but to Hep B, TT and BP when IFN- γ cytokine producing cells were greater than or equal to 10 SFU/million PBMC (≥ 10 SFU/ 10^6 PBMC) after background subtraction.

5.2.2.2 Statistical considerations

Different statistical packages were used to complete the data analysis after the ELISpot plate was read, as listed in the table below (Table 5.1).

Table 5.1: Statistical packages used for data analysis

N ^o	SOFTWARE PROGRAM	ANALYSIS TYPE	DATA TYPE
1	Microsoft Excel 2013	To clean and organize data from the ELISpot (CTL Immunospot) reader	ELISpot data analysis
2	Microsoft Excel 2013	To perform background subtraction	
3	GraphPad Prism v5 (for Windows, GraphPad Software, San Diego California USA, www.graphpad.com”).	To analyze differences in IFN- γ SFU/million cells between groups	
4	STATA v11 (for windows, StataCorp LP, College station, TX77845, USA).	Statistical tests for cohort description	Graphics -(Whole CBMC/PBMC vs. CD15) -(HEU vs. HU)

5.2.2.3 Statistical data analysis

Data analysis was performed by applying the following statistical tests;

Table 5.2: Statistical tests used to complete data analysis

Statistical test	Rationale	Statistical package	Data type
Chi-square test	Relationship between two categorical variables	STATA v11	Cohort description (HEU vs. HU)
Fisher's exact test	Relationship between two categorical variables (one or more of the values in the cells has an expected frequency ≤ 5)	STATA v11	Cohort description (HEU vs. HU)
Wilcoxon rank sum test (Mann-Whitney test)	Relationship between two continuous variables	- GraphPad Prism v5 - STATA v11	IFN- γ SFU/million cells (CD15 ⁺ vs. CD15 ⁻) (HEU vs. HU)
Wilcoxon matched-pairs sign rank test	Compare continuous data between two matched categories that is not normally distributed.	- GraphPad Prism v5	-T cell Proliferation (CD15 ⁺ vs. CD15 ⁻)
Spearman correlation	Correlations between two continuous non-normally distributed variables	- GraphPad Prism v5	(MDSC frequencies vs. immune responses)

5.3 Results

In this chapter we assessed the effect of MDSC on IFN- γ responses to BCG, *B. pertussis* (BP), TT and Hep B vaccination, with PHA as a positive control mitogen. We compared responses between whole CBMC and/or PBMC with versus without MDSC in HEU as well as HIV-unexposed (HU) infants, and in HIV-infected (HIV+) as well as HIV-uninfected (HIV-) mothers postpartum. IFN- γ responses were assessed in infants post vaccination from birth through 6, 10 and 14 weeks, 6 and 9 months, to one year of age; and in mothers only at delivery after gestation and one year postpartum.

In this chapter, *we hypothesized that in infants and pregnant women, IFN- γ production in response to vaccines will be low due to the presence of MDSC, and that, the effect in HEU infants as well as HIV+ mothers will be increased compared to the HU infants and HIV-mothers respectively.*

To clearly understand the effect of MDSC on INF- γ production, we employed two analytical processes;

1. We performed binary analyses by comparing MDSC frequency as percentage of PBMC as well as MDSC absolute counts per million PBMC of infants in responders or non-responders based on the quantity of INF- γ cytokine producing cells to antigens (section 5.2.2.1). The number of INF- γ cytokine producing cells in response to vaccine antigens varied between antigens to be considered as responder or non-responders. No consensus has been reached as of now on how to determine if an immune response has been detected based on the raw data from an ELISpot assay (Moodie *et al.*, 2010).
2. (a) We also compared INF- γ cytokine producing cells to vaccines and antigens in whole PBMC (presence of MDSC) versus without MDSC (after MDSC depletion) at all individual time points post vaccination (i.e. at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age), and in mothers (i.e. at delivery and one year postpartum).
 (b) In infants, we performed grouped analyses, based on the time points the different vaccines were administered and followed-up during infancy, to increase the sample numbers. BP, TT and HepB vaccinations were evaluated in two groups; at 10 and 14 weeks (after the first and second vaccine doses and before the last vaccine doses), and at 6 and 9 months and one year (after the last vaccine doses and the last follow-up time points where peak responses occur). However, for BCG follow-up ages were grouped into two, at 6, 10 and 14 weeks (first few weeks after priming), and at 6 and 9 months and one year (the last follow-up time points).

Generally, after vaccine stimulation at most time points there were very low IFN- γ spot forming cells and vaccine responses varied in the different antigens through time. Some responses to vaccines were stronger and some low. [Appendix 5.2](#) shows the general ontogeny of each vaccine response (median counts of IFN- γ cytokine producing cells) to the different antigens in infants and mothers through time.

Hep B responses were low at 10 and 14 weeks after priming at 6 weeks in infants and remained low till 6 months of age. The responses then peaked at 9 months to one year of age [[Appendix 5.2, Figure U\(i\)](#)]. In the mothers, [Figure U\(ii\)](#), Hep B responses were higher at one year postpartum than at delivery.

Generally, TT responses were low throughout from after priming at 6 weeks of age to one year of age [[Appendix 5.2, Figure V\(i\)](#)]. In the mothers, [Figure V\(ii\)](#), TT responses dropped from delivery compared to one year postpartum.

BP responses were generally low throughout, and varied with age in infants [[Appendix 5.2, Figure W\(i\)](#)]. In the mothers, [Figure W\(ii\)](#), BP responses were low at delivery and at one year postpartum.

BCG responses peaked at birth to 6, 10 and 14 weeks after BCG vaccination at birth in infants as previously described ([Kagina et al., 2009](#)) [[Appendix 5.2, Figure X\(i\)](#)]. The responses then dropped to lower levels at 9 months to one year of age comparable to peak responses between 6 weeks and 6 months of age [Figure X\(i\)](#). In the mothers, [Figure X\(ii\)](#), BCG responses were higher at delivery compared to one year postpartum.

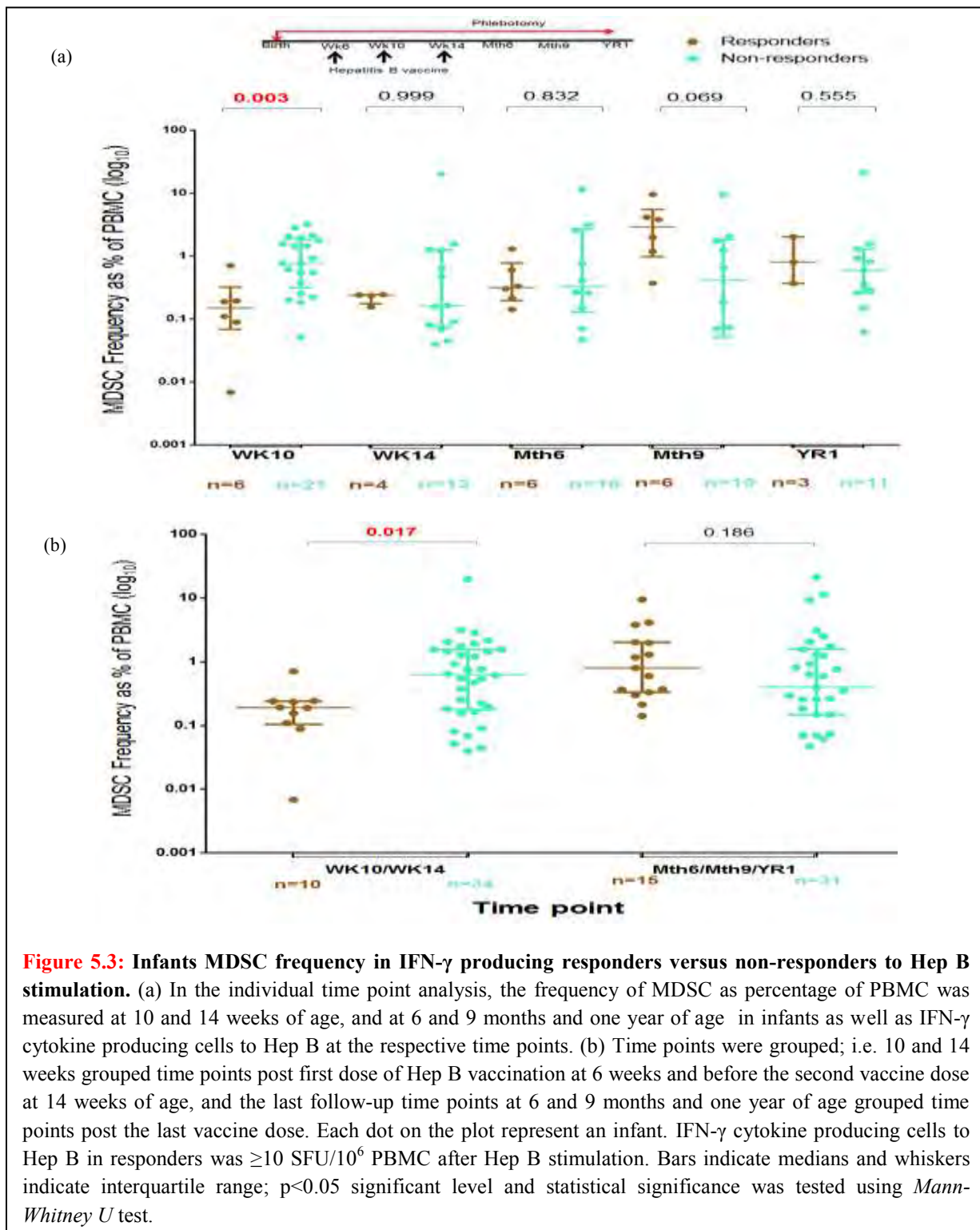
As expected of a positive control and strong stimulation, PHA responses were strong from birth through 6, 10 and 14 weeks in infants till 6 and 9 months of age. [[Appendix 5.2, Figure Y\(i\)](#)]. In mothers, [Figure Y\(ii\)](#), PHA responses were high at delivery as well as at one year postpartum.

5.3.1 Infant MDSC frequency and absolute counts in IFN- γ producing responders versus non-responders to vaccines stimulation

In [section 4.3.1](#) we looked at the relationship between the frequency and absolute count of MDSC and the frequency of CD4⁺ T cell proliferative responders versus non-responders to vaccines and antigens at the time at which responses were measured. Here, we evaluated the association between the frequency and absolute count of MDSC and the total spot forming IFN- γ cytokine producing cells i.e. net spot forming units per million PBMC (Net SFU/10⁶ PBMC) in responders compared to non-responders to vaccines and antigens post vaccination to one year of age in infants. Further, we evaluated maternal MDSC frequency in IFN- γ cytokine producing responders versus non-responders at delivery and one year postpartum to vaccine and antigen stimulations. [Table 5.3](#) below shows the summary of the results.

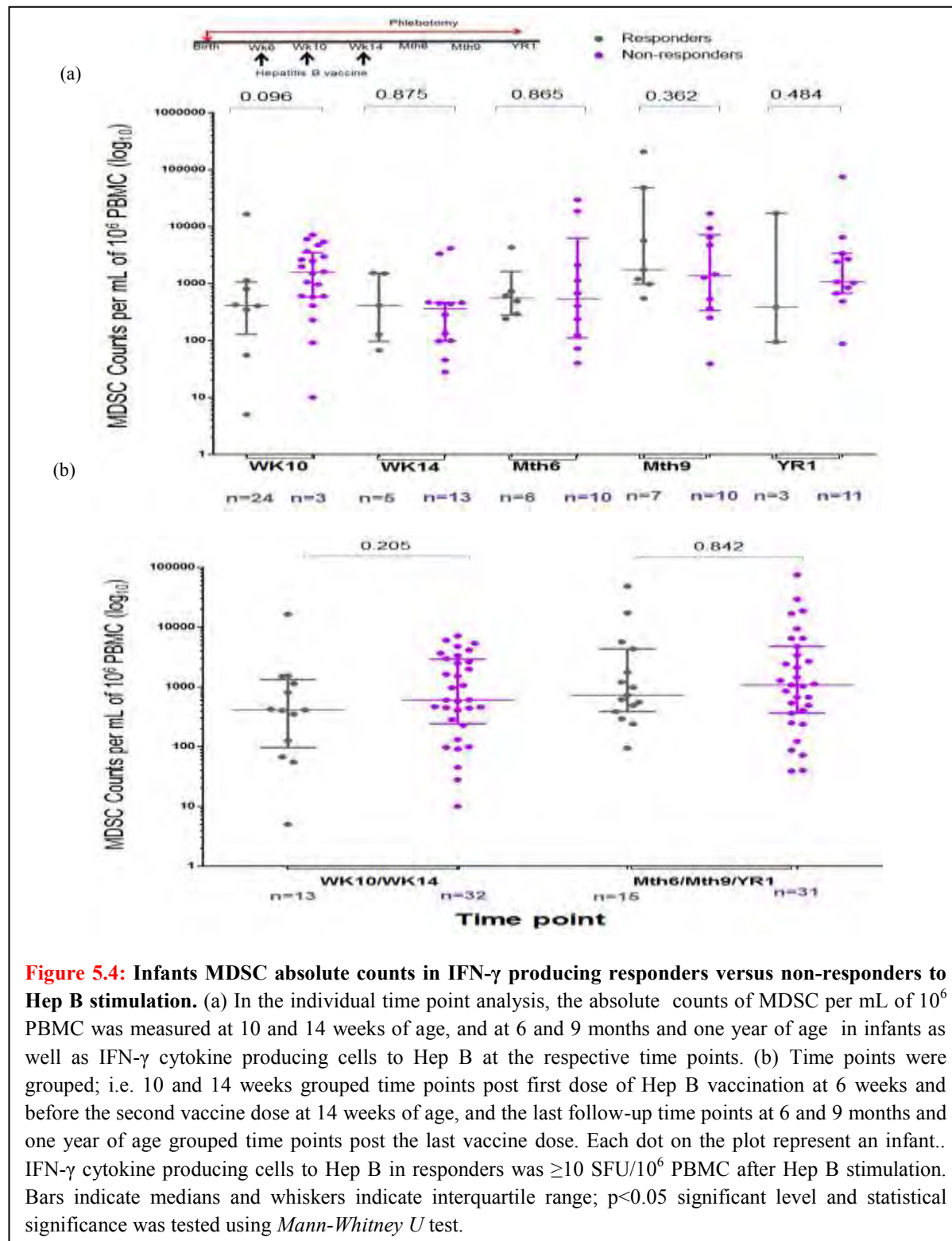
5.3.1.1 Infant MDSC frequency in IFN- γ responders versus non-responders to Hep B stimulation

At the individual time points post Hep B vaccination, the frequency of MDSC after Hep B stimulation in IFN- γ cytokine producing PBMC from infants who responded at 10 weeks of age was significantly lower compared to the MDSC frequency in the non-responders (median of responders=0.149% versus non-responders=0.770% and $p=0.003$) **Figure 5.3 (a)**. No significant differences were observed at the other time points between responder versus non-responders at 14 weeks of age ($p=0.999$), as well as at 6 months and one year of age ($p=0.832$ and $p=0.555$ respectively). However, at 9 months of age, there was a trend towards high MDSC frequency in responders compared to the non-responders ($p=0.069$), but was not significant (**Figure 5.3**). At the grouped time points analyses, the frequency of MDSC after Hep B vaccine stimulation in infants who responded versus non-responders of IFN- γ cytokine responses was measured in the grouped time point at 10 and 14 weeks grouped time points, and at 6 and 9 months and one year of age grouped time points. As shown in **Figure 5.3 (b)**, responders had a significantly lower MDSC frequency at 10 and 14 weeks grouped time points compared to non-responders (median of responders=0.192% versus non-responders=0.634% and $p=0.017$), but was not significantly different at 6 and 9 months and one year grouped time points (median of responders=0.802% versus non-responders=0.404% and $p=0.186$).



5.3.1.2 Infants MDSC absolute counts in IFN- γ responders versus non-responders to Hep B stimulation

We evaluated the absolute count of MDSC after Hep B vaccine stimulation in infants who responded versus non-responders to Hep B induced IFN- γ cytokine producing cells. As shown in **Figure 5.4 (a)** in the individual time points there were no significant differences between the absolute count of MDSC in responders at 14 weeks ($p=0.875$), 6 months ($p=0.865$), 9 months ($p=0.362$) and 1 year of age ($p=0.484$) of age. However, at 10 weeks of age post first vaccine dose, there was a trend towards high MDSC absolute counts in infants who had poor IFN- γ cytokine producing cell to Hep B stimulation compared to responders (median of responders=412.5 MDSC counts versus non-responders=1567.0 MDSC counts and $p=0.096$) **Figure 5.4 (a)**. The absolute counts of MDSC after Hep B vaccine stimulation in infants who responded compared to infants who responded poorly to Hep B induced IFN- γ cytokine producing cells was measured in the grouped time point analysis at 10 and 14 weeks, and at 6 and 9 months and one year of age. There were no significant differences between responders and non-responders MDSC counts at all grouped time points at 10 and 14 weeks ($p=0.205$), and at 6 and 9 months and one year ($p=0.842$) **Figure 5.4 (b)**.



5.3.1.3 Infant MDSC frequency in IFN- γ responders versus non-responders to TT stimulation

Due to the limited number of samples for which IFN- γ producing cells were to be measured at the individual time points, we compared MDSC frequency of infants who were responders versus non-responders to TT induced IFN- γ production at grouped time points. The frequency of MDSC in infants who responded versus non-responders to TT induced IFN- γ SFU/ 10^6 PBMC was measured in the grouped time point analysis at 10 and 14 weeks, and at 6 and 9 months and one year of age. As shown in **Figure 5.5**, responders had a significantly higher MDSC frequency at 6 and 9 months and one year grouped time points (median of responders=1.250% versus non-responders=0.359% and $p=0.036$), but there was a trend towards high MDSC frequency in non-responders at 10 and 14 weeks grouped time points that was not significant (median of responders=0.189% versus non-responders=0.619% and $p=0.074$) (**Figure 5.5**).

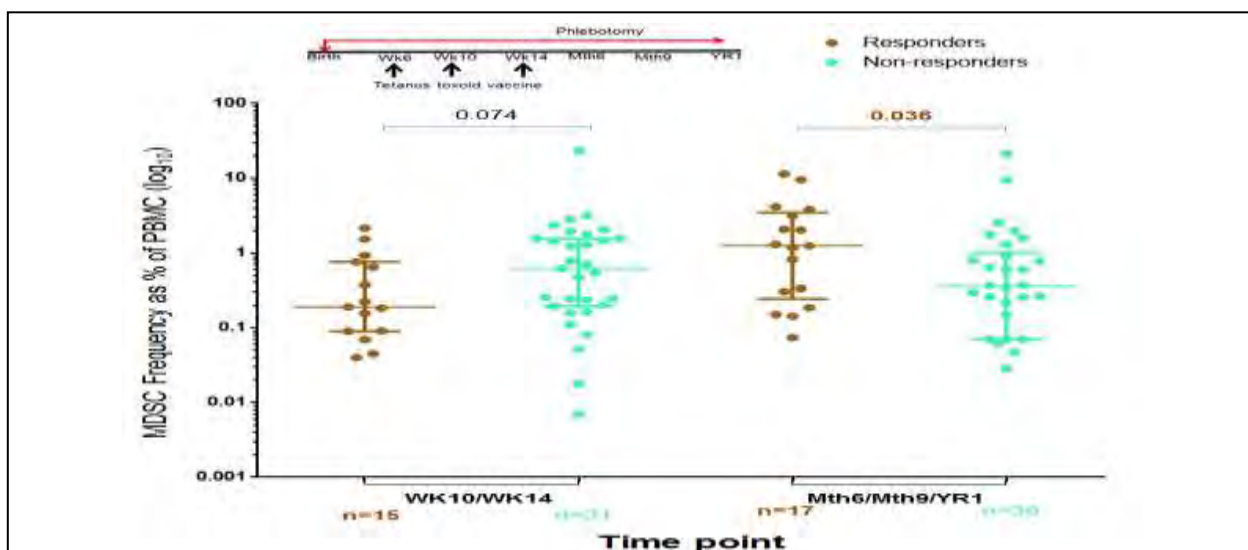


Figure 5.5: Infants MDSC frequency in IFN- γ producing responders versus non-responders to TT stimulation. The frequency of MDSC as percentage of PBMC was measured at 10 and 14 weeks of age, and at 6 and 9 months and one year of age in infants as well as IFN- γ cytokine producing cells to TT at the respective time points. Time points were grouped; i.e. 10 and 14 weeks grouped time points post first dose of TT vaccination at 6 weeks and before the second vaccine dose at 14 weeks of age, and the last follow-up time points at 6 and 9 months and one year of age grouped time points post the last vaccine dose. Each dot on the plot represent an infant. IFN- γ cytokine producing cells to TT in responders was ≥ 10 SFU/ 10^6 PBMC after TT stimulation. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

5.3.1.4 Infant MDSC absolute counts in IFN- γ producing responders versus non-responders to TT stimulation

Due to the limited number of samples for which IFN- γ producing cells were to be measured at the individual time points, we compared MDSC absolute counts of infants who were responders versus non-responders to TT induced IFN- γ production at grouped time points. The absolute counts of MDSC in infants who responded compared to infants produced low or undetectable IFN- γ to TT were measured in the grouped time point analysis at 10 and 14 weeks, and at 6 and 9 months and one year of age. At 10 and 14 weeks grouped time points, there was a significantly higher MDSC absolute counts in the non-responders compared to the responder of IFN- γ cytokine producing cells to TT (median of responders=128.5 MDSC counts versus non-responders=1150.0 MDSC counts and $p=0.005$), but the difference was not significant at 6 and 9 months and one year ($p=0.213$) (Figure 5.6).

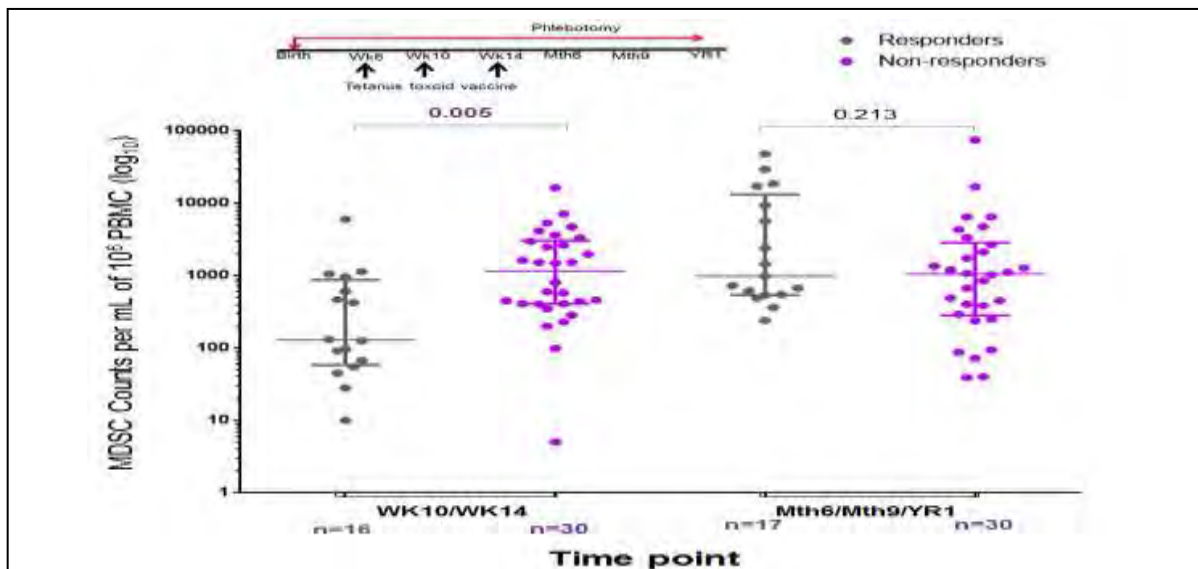


Figure 5.6: Infants MDSC absolute counts in IFN- γ producing responders versus non-responders to TT stimulation. The absolute counts of MDSC per mL of 10^6 PBMC was measured at 10 and 14 weeks of age, and at 6 and 9 months and one year of age in infants as well as IFN- γ cytokine producing cells to TT at the respective time points. Time points were grouped; i.e. 10 and 14 weeks grouped time points post first dose of TT vaccination at 6 weeks and before the second vaccine dose at 14 weeks of age, and the last follow-up time points at 6 and 9 months and one year of age grouped time points post the last vaccine dose. Each dot on the plot represent an infant. IFN- γ cytokine producing cells to TT in responders was ≥ 10 SFU/ 10^6 PBMC after TT stimulation. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

5.3.1.5 Infant MDSC frequency in IFN- γ responders versus non-responders to BP stimulation

Due to the limited number of samples for which IFN- γ producing cells were to be measured at the individual time points, we compared MDSC frequency of infants who were responders versus non-responders to BP induced IFN- γ production at grouped time points. The frequency of MDSC in infants who responded (≥ 10 SFU/ 10^6 PBMC) versus non-responders to BP was measured in the grouped time point analysis at 10 and 14 weeks grouped time points, and at 6 and 9 months and one year of age grouped time points. As shown in **Figure 5.7**, there were no significant differences in MDSC frequency between responders and non-responders at 10 and 14 weeks grouped time points that was not significant ($p=0.536$), and also at 6 and 9 months and one year grouped time points ($p=0.801$) (**Figure 5.7**).

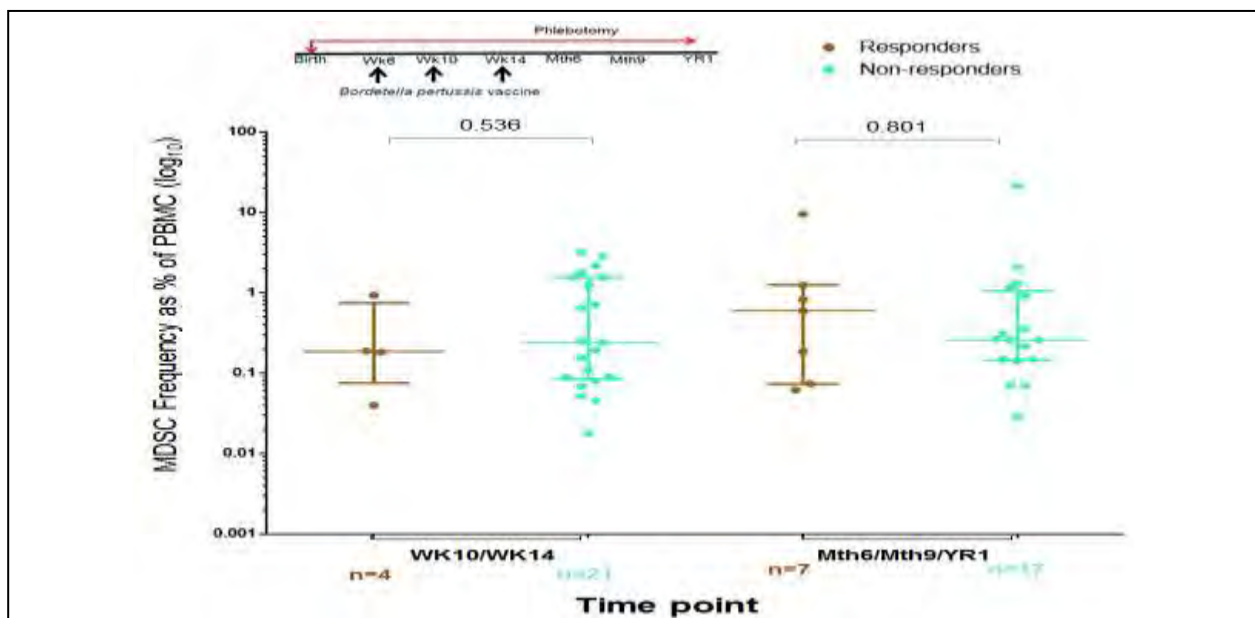
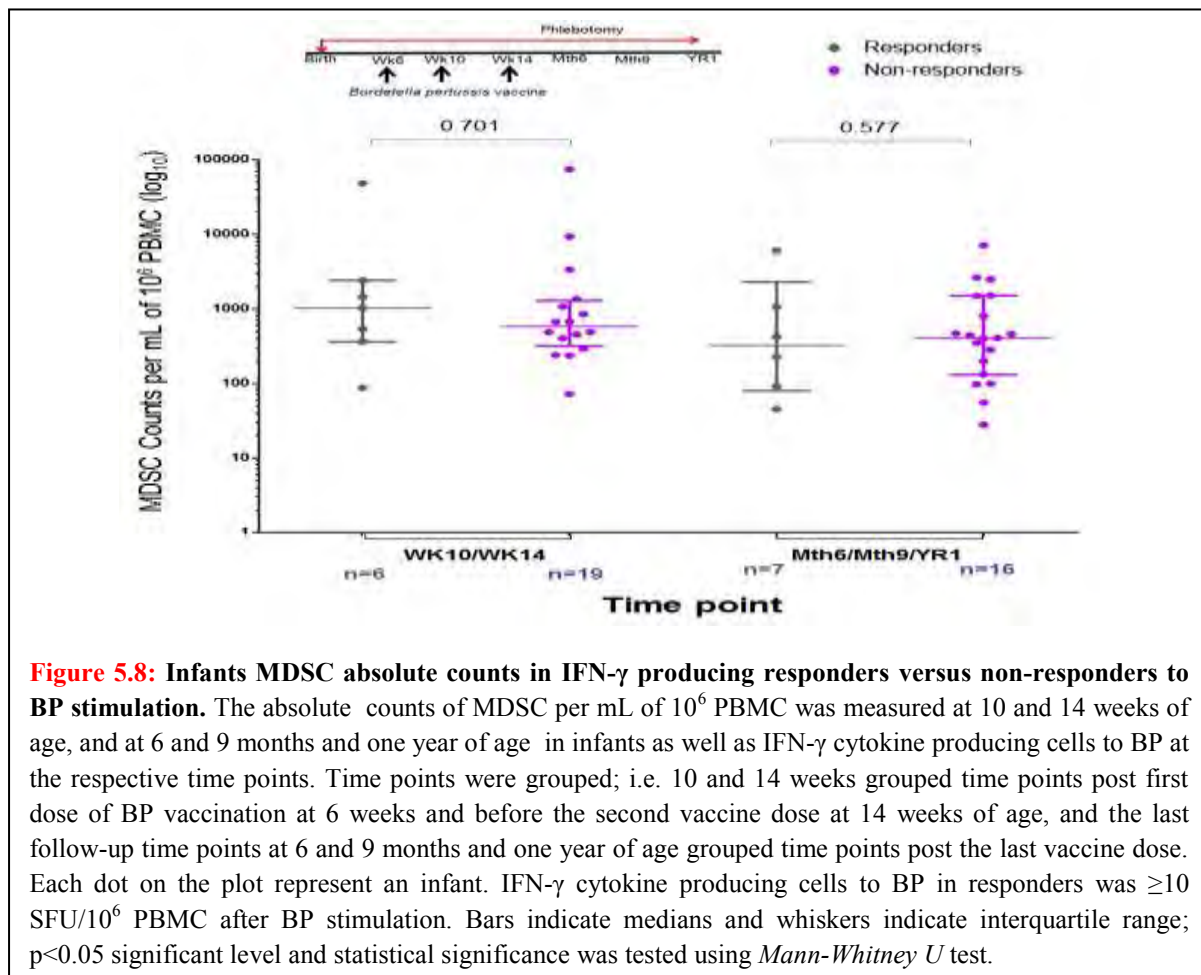


Figure 5.7: Infants MDSC frequency in IFN- γ producing responders versus non-responders to BP stimulation. The frequency of MDSC as percentage of PBMC was measured at 10 and 14 weeks of age, and at 6 and 9 months and one year of age in infants as well as IFN- γ cytokine producing cells to BP at the respective time points. Time points were grouped; i.e. 10 and 14 weeks grouped time points post first dose of BP vaccination at 6 weeks and before the second vaccine dose at 14 weeks of age, and the last follow-up time points at 6 and 9 months and one year of age grouped time points post the last vaccine dose. Each dot on the plot represent an infant. IFN- γ cytokine producing cells to BP in responders was ≥ 10 SFU/ 10^6 PBMC after BP stimulation. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

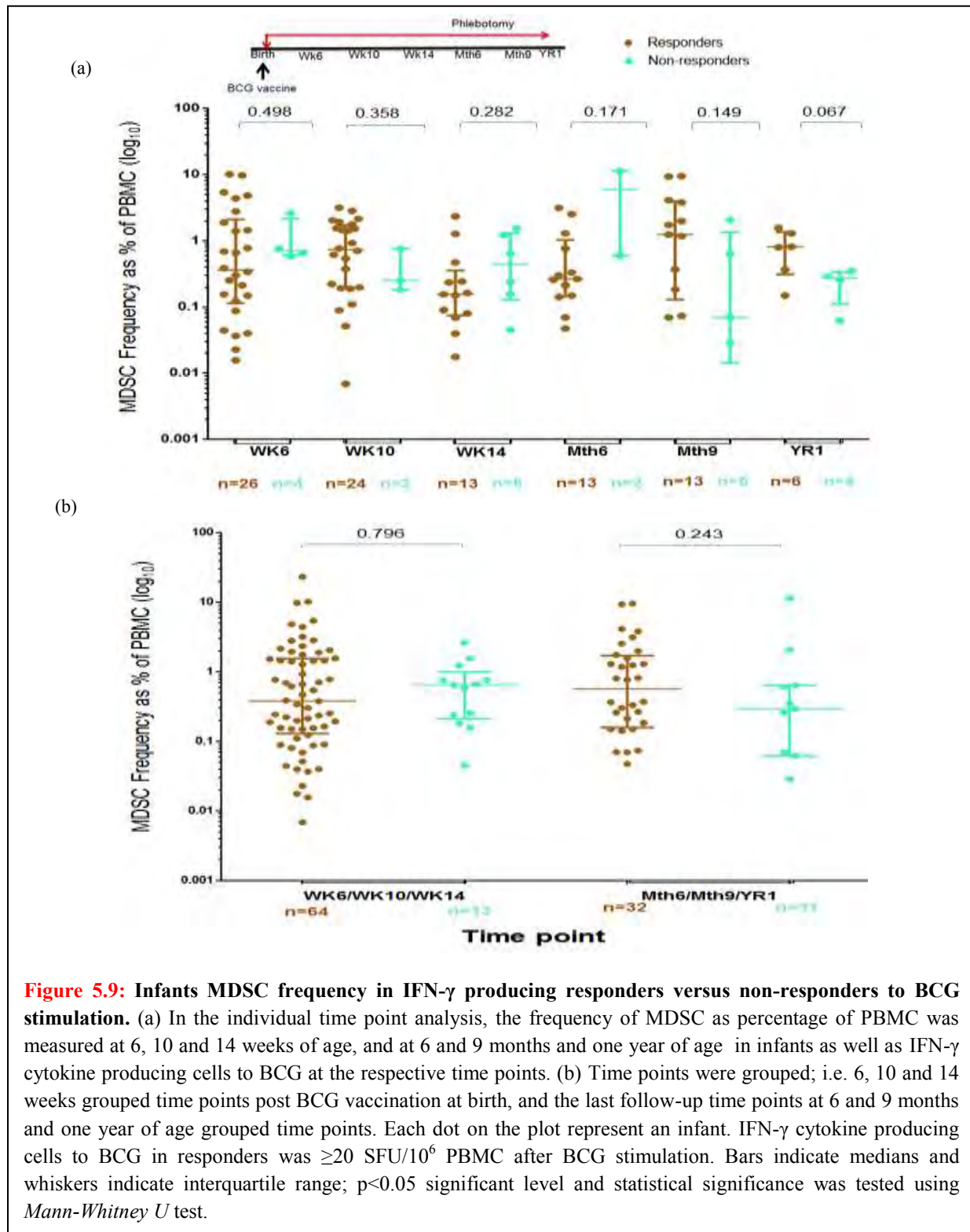
5.3.1.6 Infants MDSC absolute counts in IFN- γ responders versus non-responders to BP stimulation

Due to the limited number of samples for which IFN- γ producing cells were to be measured at the individual time points, we compared MDSC absolute counts of infants who were responders versus non-responders to BP induced IFN- γ production at grouped time points. The absolute counts of MDSC in infants who responded compared to infants who were poor producers of IFN-g to BP was measured in the grouped time point analysis at 10 and 14 weeks grouped time points, and at 6 and 9 months and one year of age grouped time points. There were no significant differences MDSC absolute counts between responders and non-responders at 10 and 14 weeks grouped time points ($p=0.701$), as well as at 6 and 9 months and one year grouped time points ($p=0.577$) (Figure 5.8).



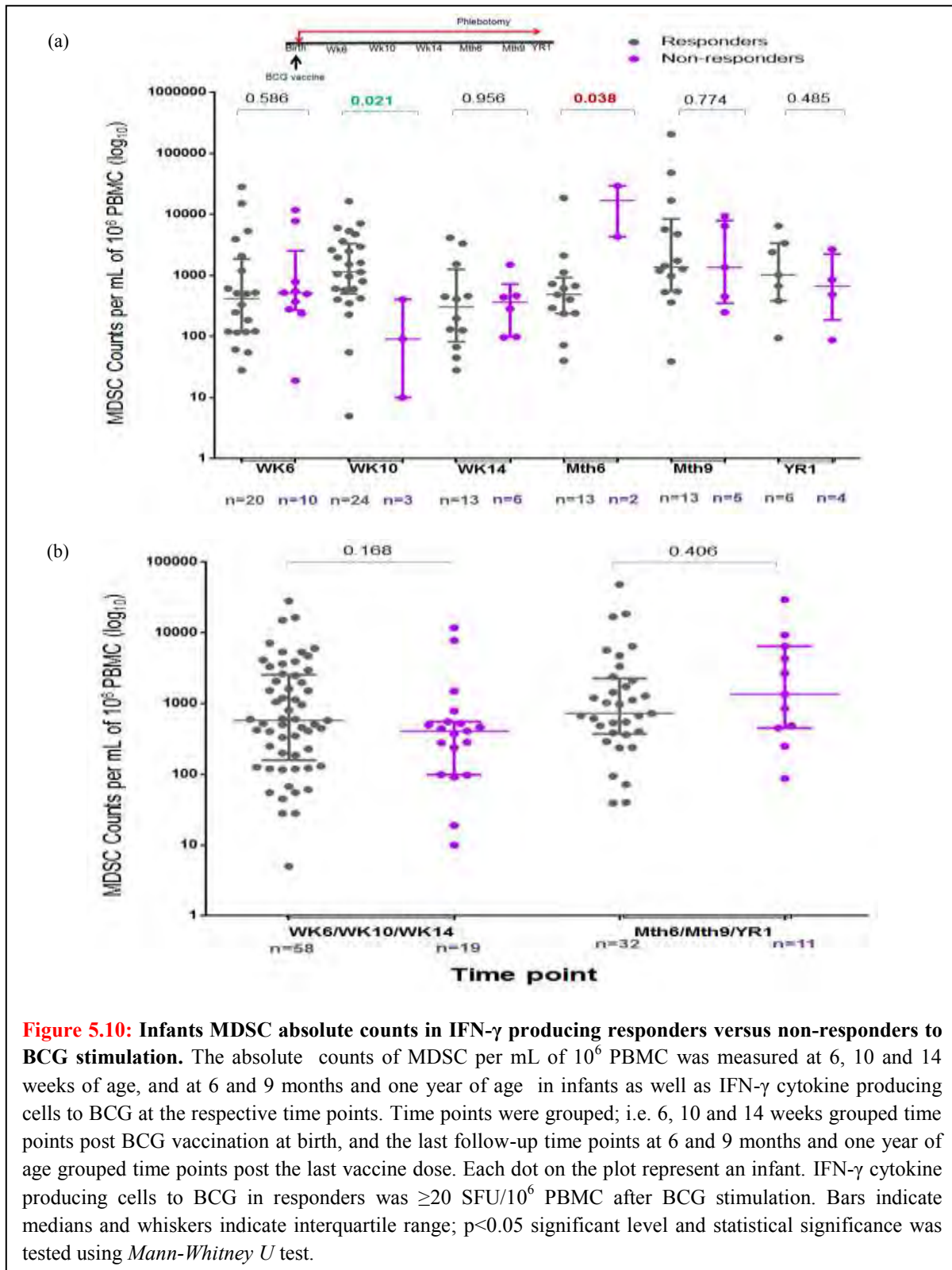
5.3.1.7 Infants MDSC frequency in IFN- γ producing responders versus non-responders to BCG stimulation

At the individual time points post BCG vaccination at birth, the frequency of MDSC in PBMC from infants who had IFN- γ responses to BCG stimulation at 6 weeks of age was not significantly different compared to the MDSC frequency in the non-responders (median of responders=0.366% versus non-responders=0.712% and $p=0.498$) **Figure 5.9 (a)**. Also, no significant differences were observed at the other time points between responder versus non-responders at 10 and 14 weeks of age ($p=0.358$ and $p=0.282$ respectively), as well as at 6 and 9 months of age ($p=0.171$ and $p=0.149$ respectively). However, at one year of age, there was a trend towards high MDSC frequency in responders compared to non-responders (median of responders=0.810% versus non-responders=0.276% and $p=0.067$), but this was not significant **Figure 5.9 (a)**. At the grouped time point analyses, the frequency of MDSC in infants who responded versus non-responders of IFN- γ cytokine responses after BCG vaccine stimulation was measured in the grouped time points at 6, 10 and 14 weeks, and at 6 and 9 months and one year of age grouped together. As shown in **Figure 5.9 (b)**, MDSC frequency in responders did not significantly differ to that in the non-responders at 6, 10 and 14 weeks grouped time points (median of responders=0.382% versus non-responders=0.648% and $p=0.796$), as well as at 6 and 9 months and one year grouped time points (median of responders=0.569% versus non-responders=0.293% and $p=0.243$) **Figure 5.9 (b)**.



5.3.1.8 Infants MDSC absolute counts in IFN- γ producing responders versus non-responders to BCG stimulation

We evaluated the absolute count of MDSC in infants whose IFN- γ production was considered responsive versus non-responders to BCG. As shown in **Figure 5.10 (a)** the absolute count of MDSC in responders at 10 weeks of age was significantly higher than MDSC absolute counts in non-responders (median of responders=1137 MDSC counts versus non-responders=91.000 MDSC counts and $p=0.021$), however the number of non-responders was small. On the other hand, at 6 months of age the MDSC absolute counts in the non-responders was significantly higher than in the responders, however the number of infants that did not respond was small (median of responders=487.0 MDSC counts versus non-responders=16807.0 MDSC counts and $p=0.038$). There were no significant differences at other time points at 6 and 14 weeks of age ($p=0.586$ and $p=0.956$ respectively), and at 9 months ($p=0.774$) and 1 year of age ($p=0.485$) of age **Figure 5.10 (a)**. The absolute counts of MDSC in infants who responded compared to infants who responded poorly to BCG in terms of IFN- γ producing cells was measured in the grouped time point analysis at 6, 10 and 14 weeks, and at 6 and 9 months and one year of age. The differences were not significant between responders and non-responders MDSC counts at all grouped time points at 6, 10 and 14 weeks ($p=0.168$), and at 6 and 9 months and one year ($p=0.406$) **Figure 5.10 (b)**.

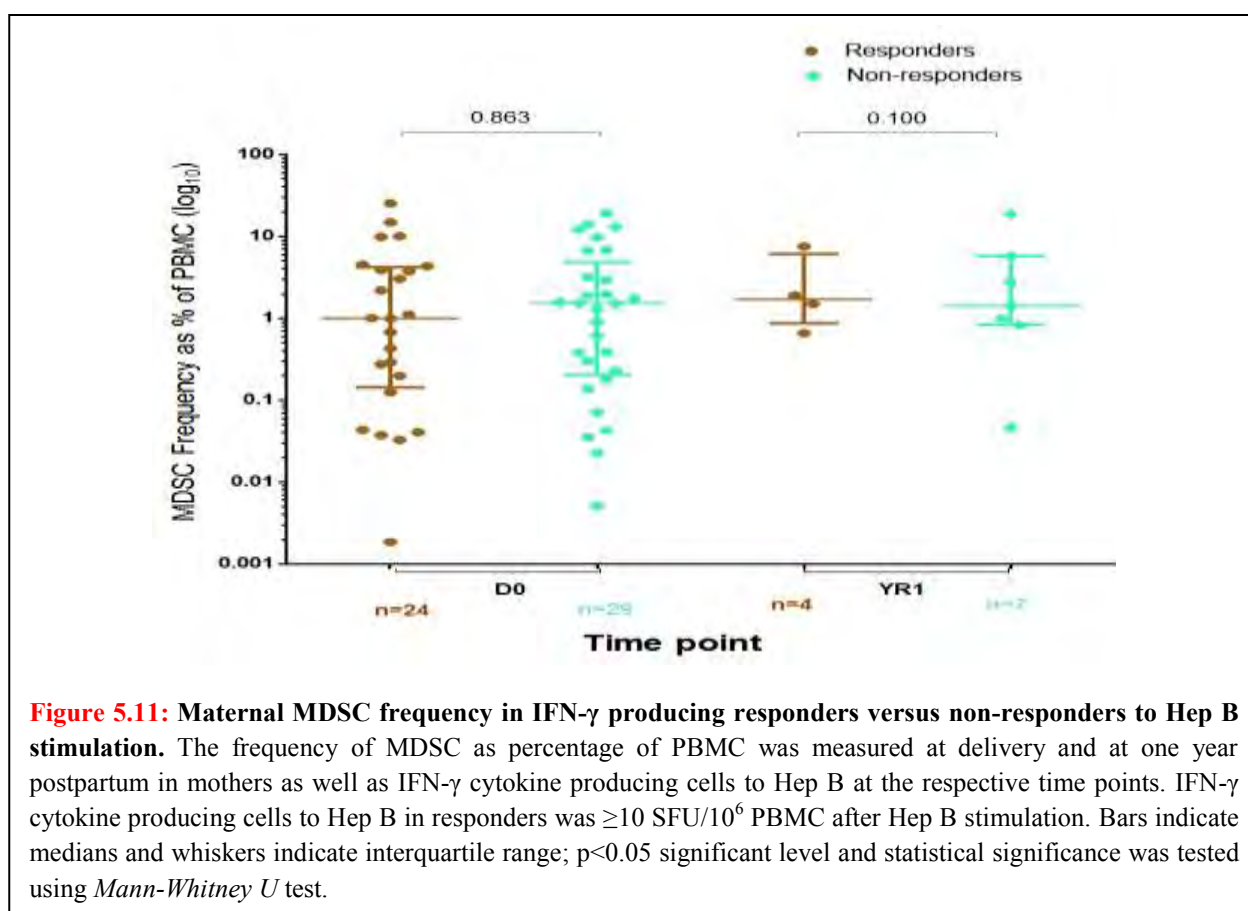


Generally, MDSC frequency and absolute count between infants who were responders and non-responders in terms of vaccine induced IFN- γ cytokine producing cells were significantly lower in responders than in non-responders at most time points, particularly to Hep B and TT.

We hypothesised that MDSC would influence IFN- γ cytokine producing cells to vaccine antigens negatively, and in support of our hypothesis in infants, this was evident. These experiments evaluated the effect of MDSC count and frequency on vaccine responses *in-vivo*. To further support our findings, we employed a more direct comparisons of IFN- γ cytokine producing cells between whole PBMC (the presence of MDSC) and in the absence of MDSC (after MDSC depletion), which would give more direct *in-vitro* evidence for or against our hypothesis (section 5.3.2). We also evaluated maternal MDSC frequency with IFN- γ responses. Table 5.3 below shows the summary of the results.

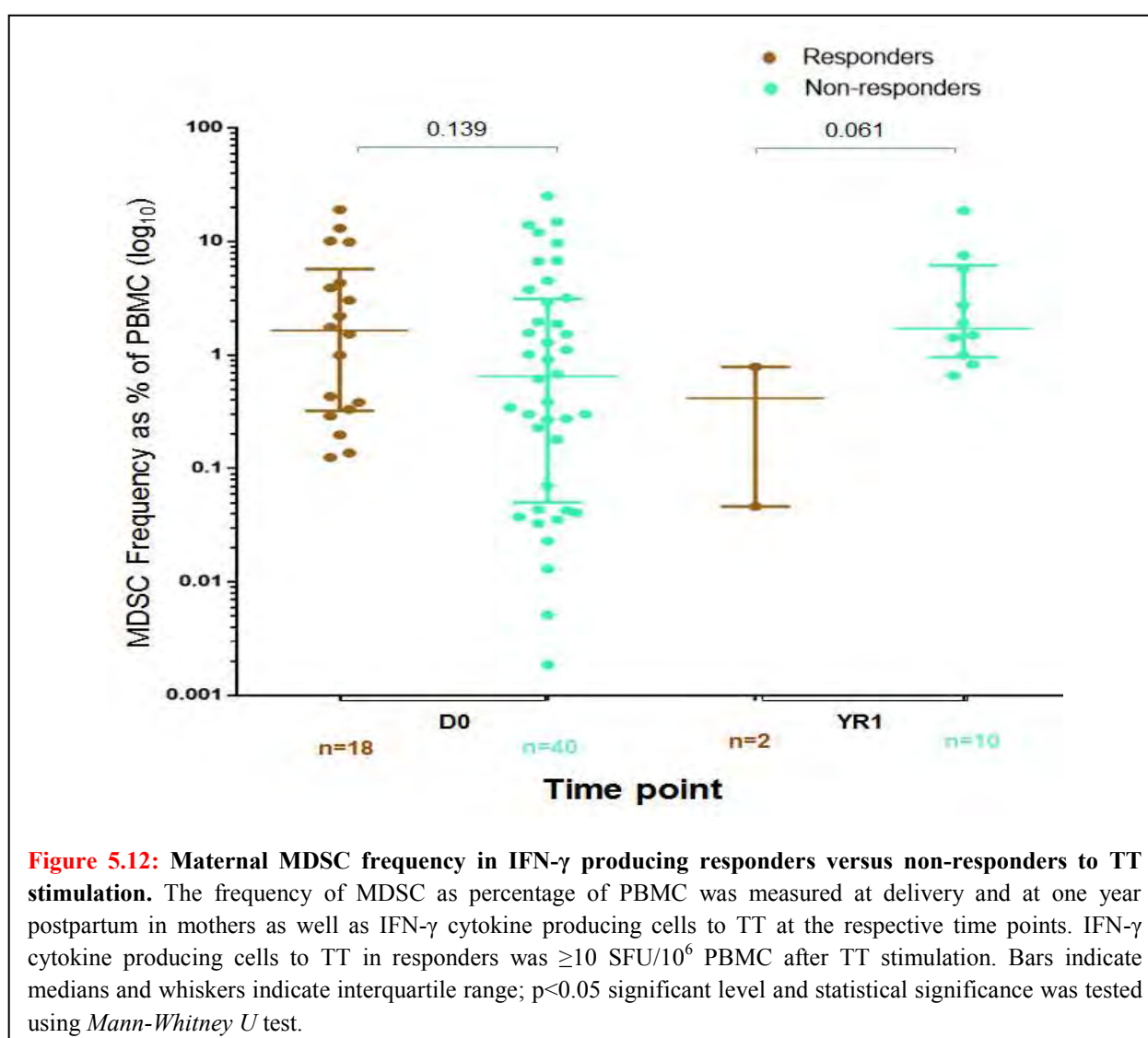
5.3.1.9 Maternal MDSC frequency in IFN- γ producing responders versus non-responders to Hep B stimulation

The frequency of maternal MDSC was not significantly different in mothers with IFN- γ cytokine producing cells ≥ 10 SFU/ 10^6 PBMC to Hep B stimulation compared to those who responded poorly with IFN- γ cytokine producing cells ≤ 10 SFU/ 10^6 PBMC. As shown in [Figure 5.11](#), there were no significant differences between maternal responders' and non-responders' MDSC frequencies at delivery (median of responders=1.003% versus non-responders=1.530% and $p=0.863$), and at one year postpartum (median of responders=1.705% versus non-responders=1.430% and $p=0.100$).



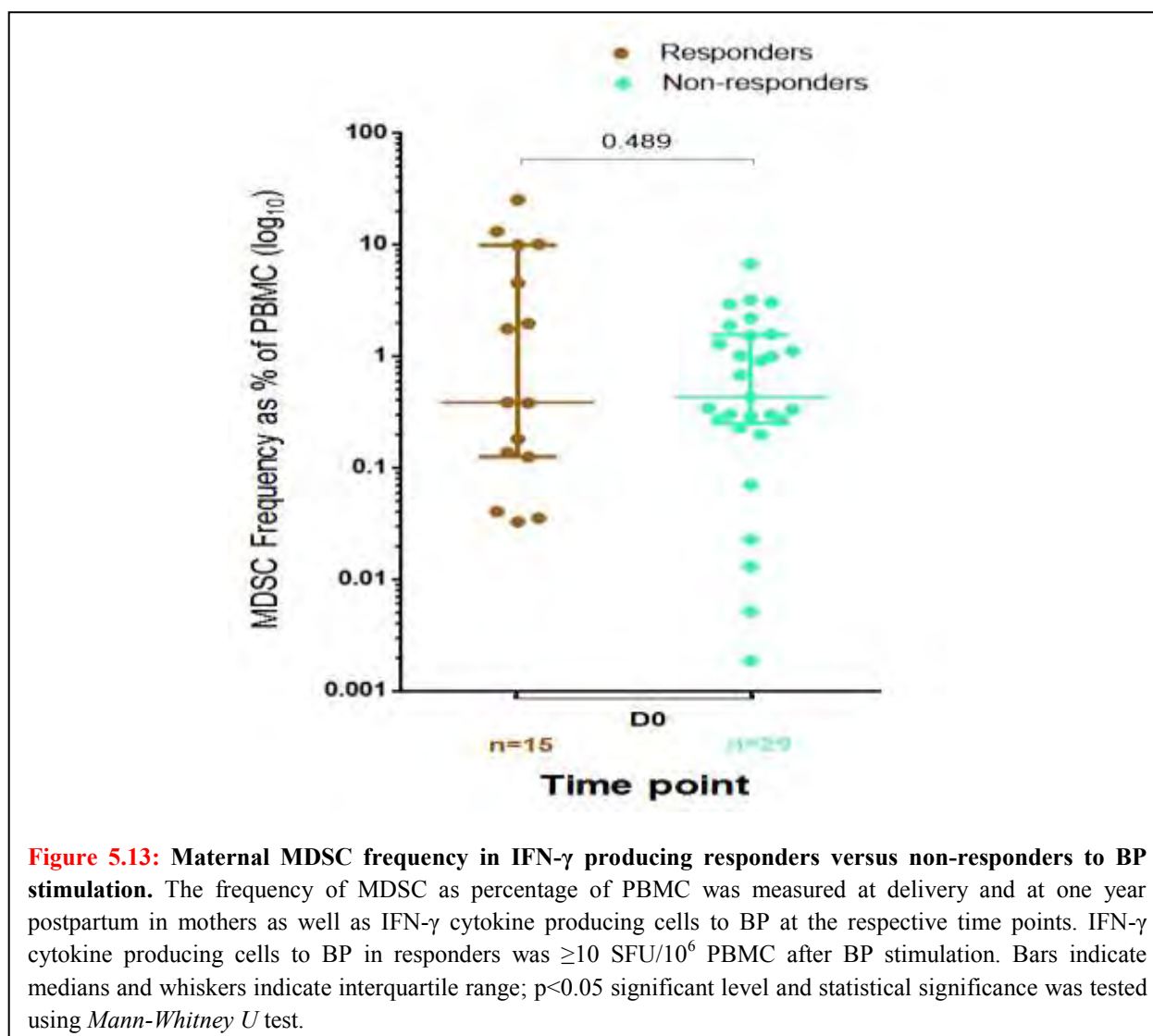
5.3.1.10 Maternal MDSC frequency in IFN- γ producing responders versus non-responders to TT stimulation

There was a trend towards a higher maternal MDSC frequency in non-responders compared to responders at one year postpartum (median of responders=0.417% versus non-responders=1.705% and $p=0.061$), but was not significant and numbers were small. At delivery difference in maternal MDSC frequency was not significant between responders and non-responders to TT stimulation (median of responders=1.645% versus non-responders=0.647% and $p=0.139$) (Figure 5.12).



5.3.1.11 Maternal MDSC frequency in IFN- γ producing responders versus non-responders to BP stimulation

At delivery, there was no significant difference in maternal MDSC frequency between responders and non-responders to BP stimulation (median of responders=0.386% versus non-responders=0.432% and $p=0.489$) (Figure 5.13). However, at one year postpartum the number of mothers for which responses were to be measured was small and statistical analyses could not be performed (data not shown).



5.3.1.12 Maternal MDSC frequency in IFN- γ producing responders versus non-responders to BCG stimulation

Maternal MDSC frequency at delivery was not significantly different in mothers who responded to BCG stimulation versus those with poor IFN- γ cytokine producing cells to BCG at birth and at one year postpartum (Figure 5.14). Maternal MDSC frequencies at delivery were median of responders=0.616% versus non-responders=1.143% and $p=0.479$, and at one year postpartum were median of responders=0.996% versus non-responders=7.55% and $p=0.100$.

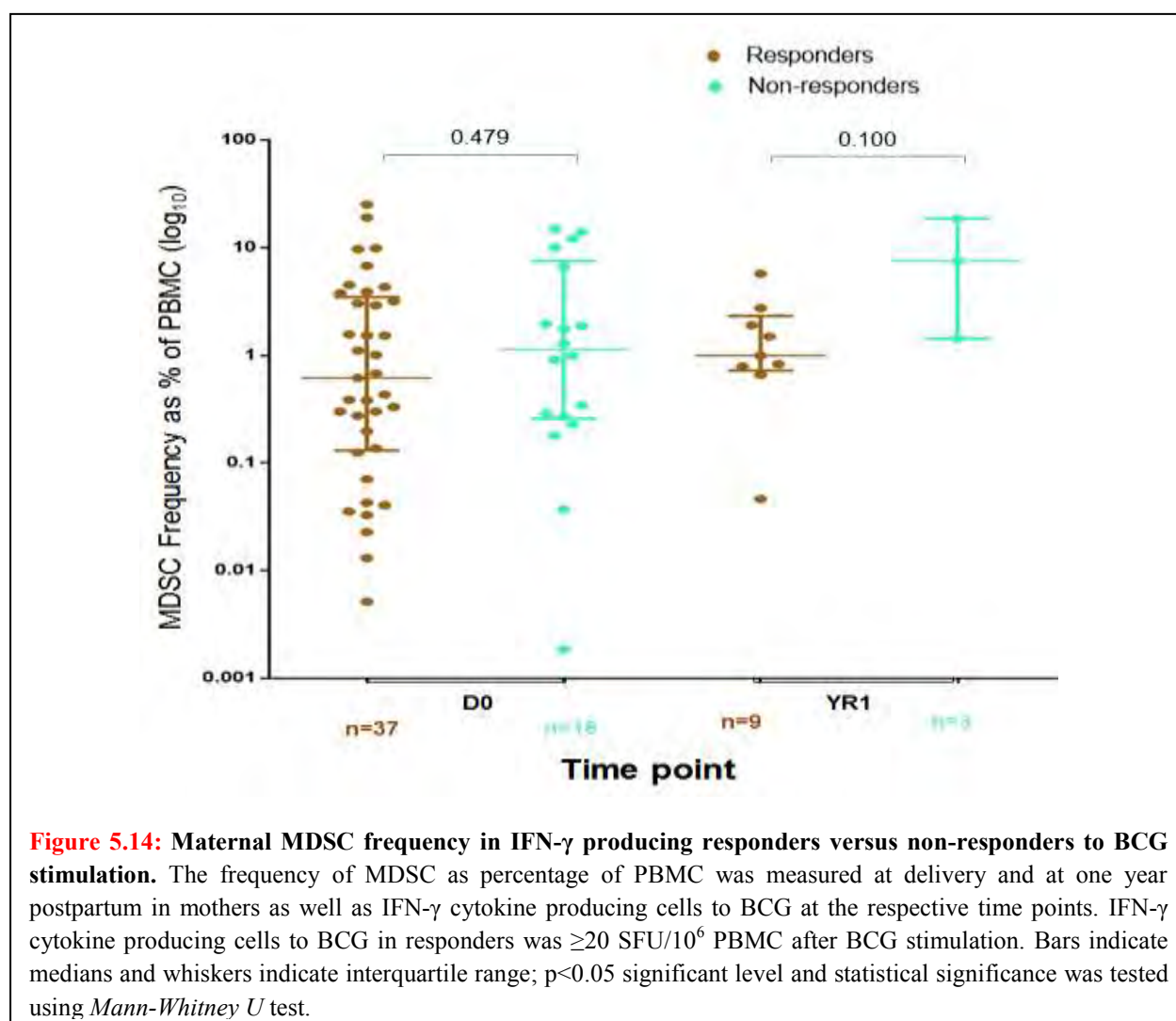


Table 5.3: Summary of the results of all infant and mothers MDSC frequency and absolute counts measured in IFN- γ producing responders versus non-responders to vaccines stimulation. P-values of infants and mothers MDSC frequency (%) and absolute count (#) measured in responders versus non-responders as measured by IFN- γ producing cells to the following antigens [Bacillus Calmette-Guérin (BCG), Hepatitis B (Hep B), Tetanus toxoid (TT) and *Bordetella pertussis* (BP)]. Blue highlights represent the parameters (p-values and type of data sets), Red highlights represent the different time points [birth (D0), 6, 10 and 14 weeks (WK6, WK10 and WK14), 6 and 9 months (Mth6 and Mth9), and 1 year (YR1)], and Purple highlights represent significant differences between MDSC quantity in responders versus non-responders to vaccine antigens. GRP=Group, Indv=Individual, TP= Time point, B=Baby, and M=Mother.

Parameters	p-values						
Time points	D0	WK6	WK10	WK14	Mth6	Mth9	YR1
Hep B Indv. TP (%MDSC) (B)			0.003	0.999	0.832	0.069	0.555
Hep B GRP TP (%MDSC) (B)			0.017		0.186		
Hep B Indv. TP (#MDSC) (B)			0.096	0.875	0.865	0.362	0.484
Hep B GRP TP (#MDSC) (B)			0.205		0.842		
TT GRP TP (%MDSC) (B)			0.074		0.036		
TT GRP TP (#MDSC) (B)			0.005		0.213		
BP GRP TP (%MDSC) (B)			0.536		0.801		
BP GRP TP (#MDSC) (B)			0.701		0.577		
BCG Indv. TP (%MDSC) (B)		0.498	0.358	0.282	0.171	0.149	0.067
BCG GRP TP (%MDSC) (B)		0.796			0.243		
BCG Indv. TP (#MDSC) (B)		0.586	0.021	0.956	0.038	0.774	0.485
BCG GRP TP (#MDSC) (B)		0.168			0.406		
Hep B Indv TP (%MDSC) (M)	0.863						0.100
TT Indv TP (%MDSC) (M)	0.139						0.061
BP Indv TP (%MDSC) (M)	0.489						
BCG Indv TP (%MDSC) (M)	0.479						0.100

5.3.2 Effect of MDSC depletion on IFN- γ production to vaccines and antigens in infants and mothers

The effect of MDSC on IFN- γ production to BCG, TT, BP, and Hep B vaccinations, and to PHA, in whole CBMC and/or PBMC were evaluated in infants and mothers in the presence of MDSC and after MDSC depletion (Figure 4.1). Since MDSC highly express CD15, as earlier described in section 4.2.1 above, CD15⁺ expressing cells were magnetically depleted from a proportion of CBMC and/or PBMC. The effect of MDSC on cells producing IFN- γ to vaccines and antigens was then measured by performing the IFN- γ ELISpot assay comparing CD15 depleted cells (absence of MDSC) and intact CBMC and/or PBMC (presence of MDSC) (Figure 4.2). In the MDSC depleted sample group, only samples with depletion purity of 90% or more were included in the analysis (Appendix 4.3).

As described in section 5.3 (2), IFN- γ cytokine producing cells were measured in infants at all individual time points post vaccination (i.e. at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age), and in mothers, the cells producing IFN- γ to vaccines and antigens were measured at delivery and again at one year postpartum. We also evaluated the ontogeny of the effect of MDSC on IFN- γ cytokine producing cells to vaccines and antigens in a grouped time points analyses, based on the time points the different vaccines assessed were administered and followed-up during infancy as described in section 5.3 (2) (b).

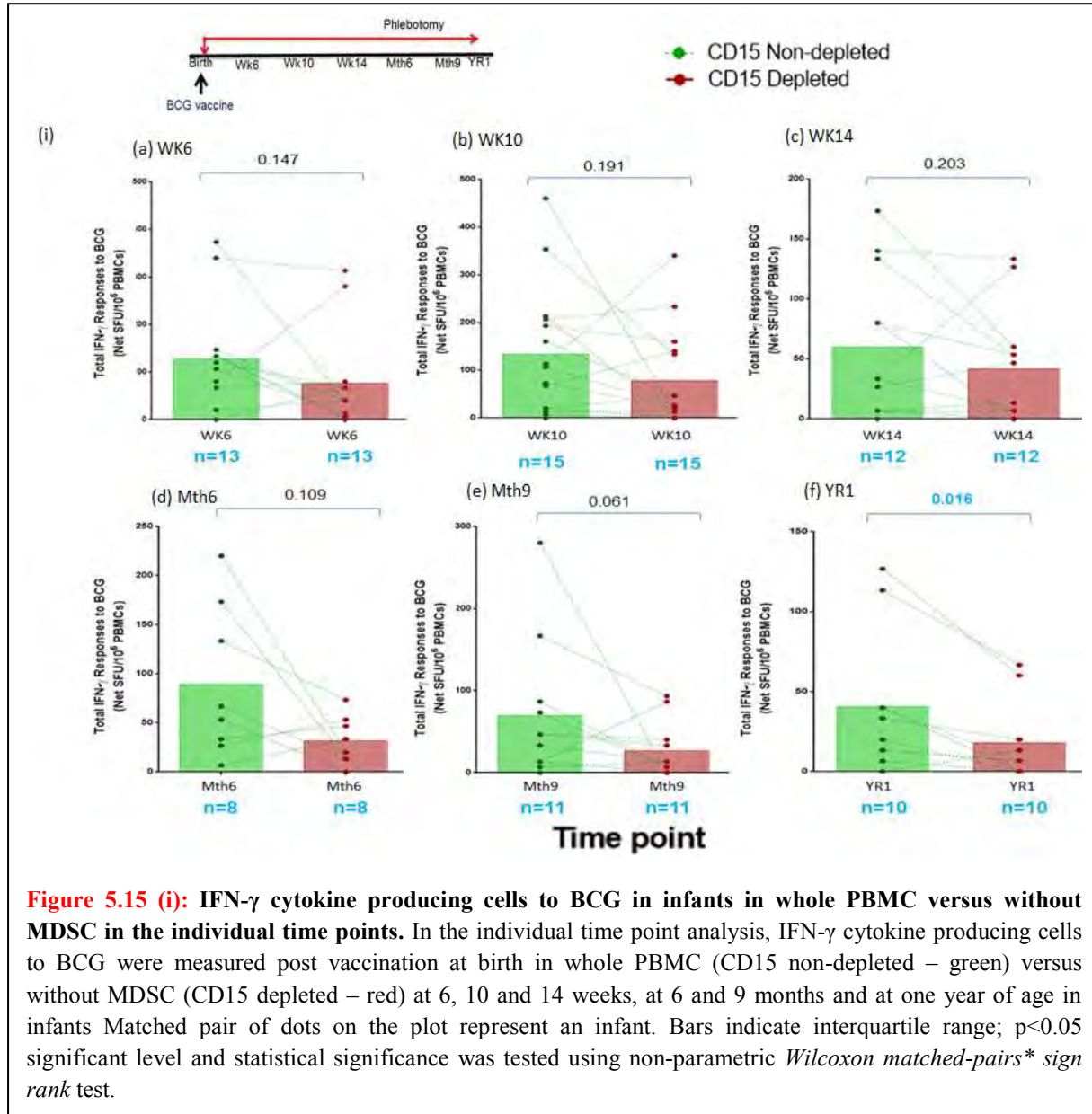
The data as presented below include matched pair of samples as whole PBMC (presence of MDSC) and the absence of MDSC (MDSC depleted). Also, unmatched comparison between HEU versus HU infants as well as HIV+ versus HIV- mothers at the respective time points either in whole PBMC (presence of MDSC) and after MDSC depletion when the number of participants were enough for statistical analyses to be performed. Table 5.4 and 5.5 below shows the summary of the results.

We prioritised experiments according to blood volume obtained, and often the volume of whole blood collected from infants was insufficient for CD15⁺ depletion. Due to this, there were only a limited number of infants and their mothers at most time points for which depletion was performed and further permitting MDSC non-depleted versus MDSC depleted

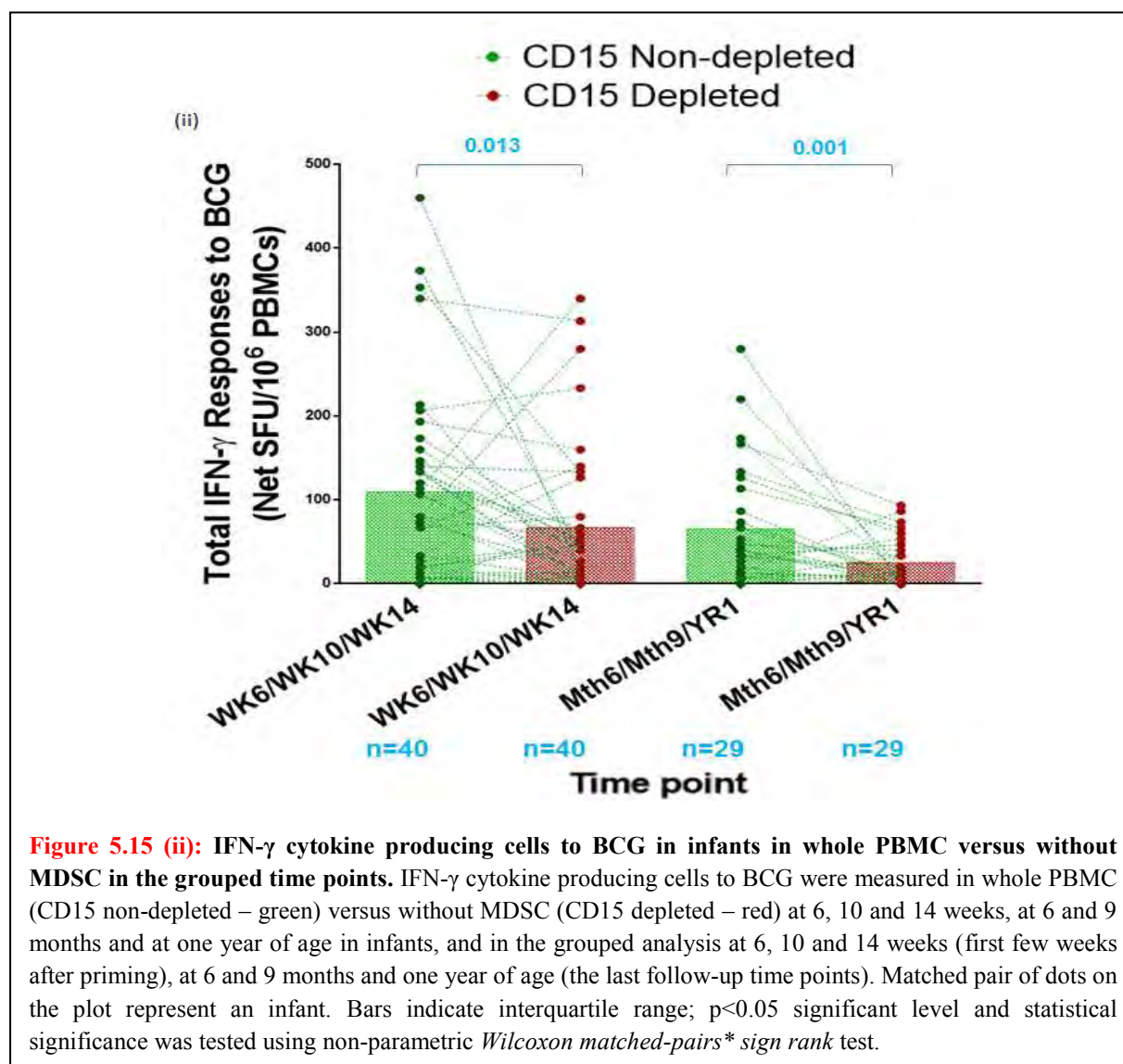
match paired analyses to be performed (see Chapter 4, Table 4.1). In Appendix 5.3 is the unmatched analyses between MDSC non-depleted versus MDSC depleted samples in all participants enrolled and from who whole blood samples was collected.

5.3.2.1 BCG induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in infants

The cells producing IFN- γ to BCG vaccination were evaluated in whole PBMC and without MDSC in infants at all time points after vaccination. As shown in Figure 5.15 there were no significant differences at most time points. However, IFN- γ cytokine producing cells to BCG



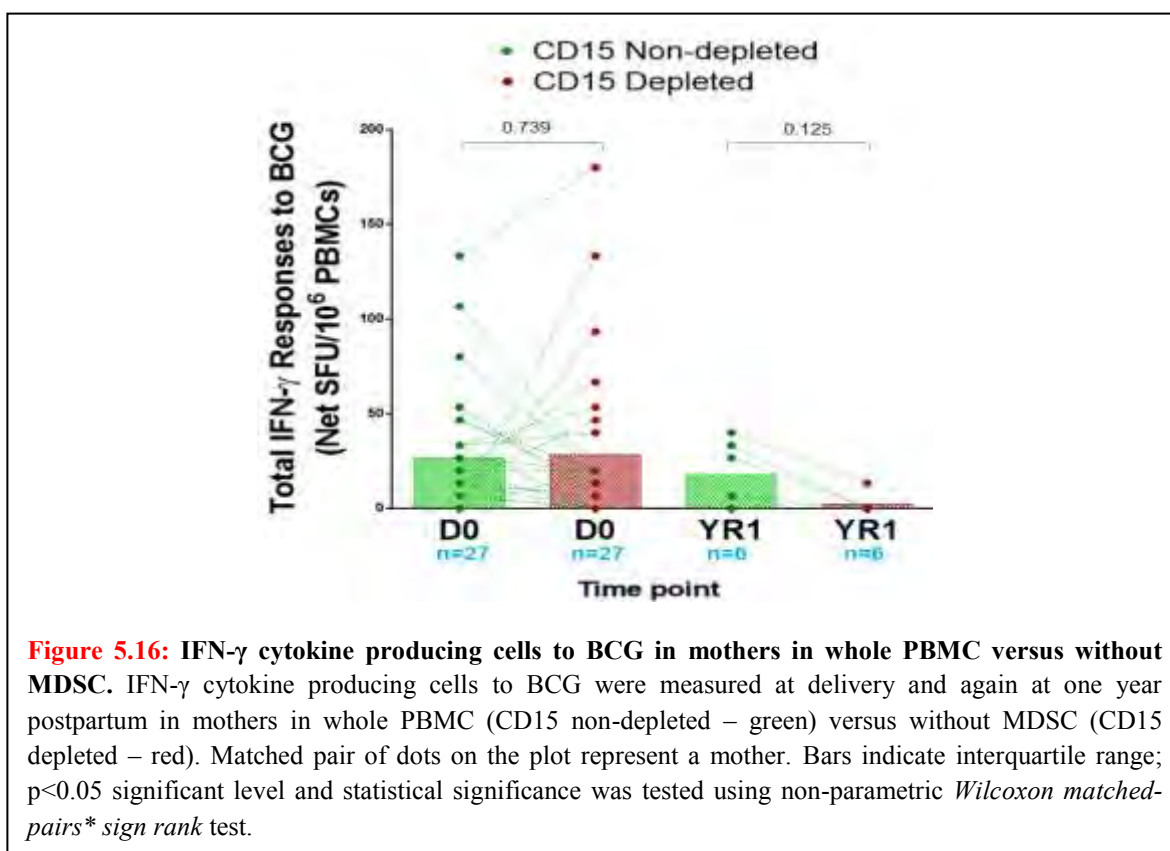
lasted till 9 months. At the individual time points at one year of age, IFN- γ cytokine producing cells to BCG were significantly higher in whole PBMC compared to after MDSC depletion in infants (median of CD15 non-depleted=26.670 Net SFU/ 10^6 PBMC versus CD15 depleted=6.667 Net SFU/ 10^6 PBMC and $p=0.016$). The differences were not significant at 6, 10 and 14 weeks ($p=0.147$, $p=0.191$ and $p=0.203$ respectively), and at 6 months of age ($p=0.109$). However, at 9 months of age there was a trend towards higher IFN- γ producing cells to BCG in whole PBMC compared to after MDSC depletion in infants (median of CD15 non-depleted=60.000 Net SFU/ 10^6 PBMC versus CD15 depleted=26.670 Net SFU/ 10^6 PBMC



and $p=0.061$) [Figure 5.15 (i)]. In the grouped time point analyses, IFN- γ cytokine producing cells to BCG were significantly higher in intact PBMC compared to after MDSC depletion at the earlier grouped time points (median of CD15 non-depleted=80.000 Net SFU/ 10^6 PBMC versus CD15 depleted=40.000 Net SFU/ 10^6 PBMC; $p=0.013$) and at 6, 9 months and 1 year grouped time points (median of CD15 non-depleted=40.000 Net SFU/ 10^6 PBMC versus CD15 depleted=13.330 Net SFU/ 10^6 PBMC; $p=0.001$) [Figure 5.15 (ii)].

5.3.2.2 BCG induced IFN- γ production in whole PBMC versus after MDSC depletion in mothers

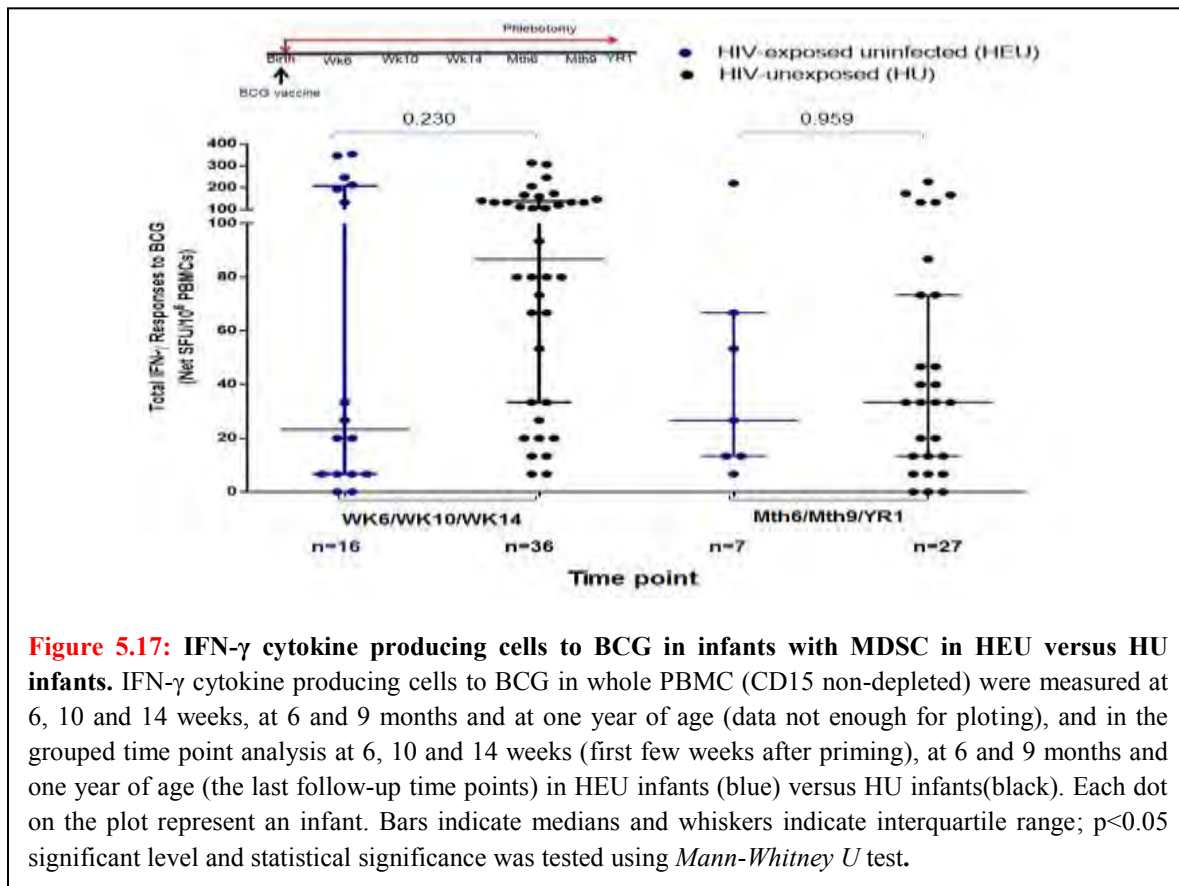
In mothers, BCG induced IFN- γ producing cells were evaluated in whole PBMC and without MDSC at delivery and at one year postpartum. As shown in Figure 5.16, the frequency of cells producing IFN- γ to BCG were not significantly different in mothers after gestation at delivery (median of CD15 non-depleted=13.330 Net SFU/ 10^6 PBMC, IQR=0-33.330 versus CD15 depleted=13.330 Net SFU/ 10^6 PBMC, IQR=0-40.000 and $p=0.839$), as well as at one year



postpartum (median of CD15 non-depleted=16.670 Net SFU/10⁶ PBMC versus CD15 depleted=0 Net SFU/10⁶ PBMC and $p=0.125$), but was not significant, and numbers in this analysis were small (Figure 5.16).

5.3.2.3 BCG induced IFN- γ cytokine producing cells in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants

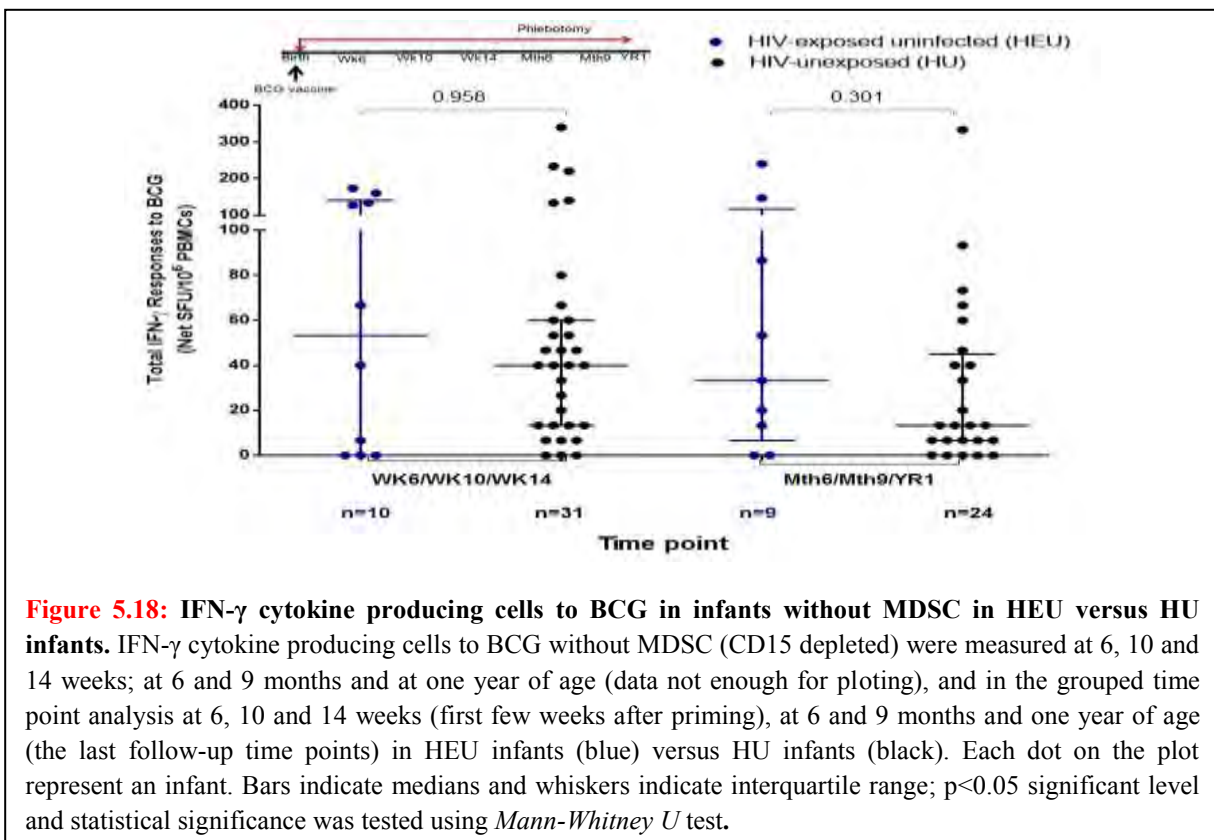
Just as we were interested in the effect of HIV-exposure on MDSC with regards to CD4⁺ T cell proliferation in chapter 4, we evaluated if HIV-exposure would alter the effect of MDSC on IFN- γ production. First, we evaluated cells producing IFN- γ to BCG in whole PBMC between HEU versus HU infants. At most time points there were no significant differences at the individual time points in cells producing IFN- γ (however, at one year, there were no samples in the HEU infants with sufficient blood for ELISPOT and statistical analysis could



not be performed; data not shown). As shown in **Figure 5.17**, in the grouped time point analysis there were no significant differences at 6, 10 and 14 weeks grouped together in IFN- γ producing cells to BCG between HEU compared to HU infants (median of HEU=24.867 Net SFU/ 10^6 PBMC versus HU=83.670 Net SFU/ 10^6 PBMC; $p=0.230$), or at 6, 9 months and one year (median of HEU=29.330 Net SFU/ 10^6 PBMC versus HU=33.670 Net SFU/ 10^6 PBMC; $p=0.959$) (**Figure 5.17**).

5.3.2.4 BCG induced IFN- γ cytokine producing cells after MDSC depletion in HEU versus HU infants

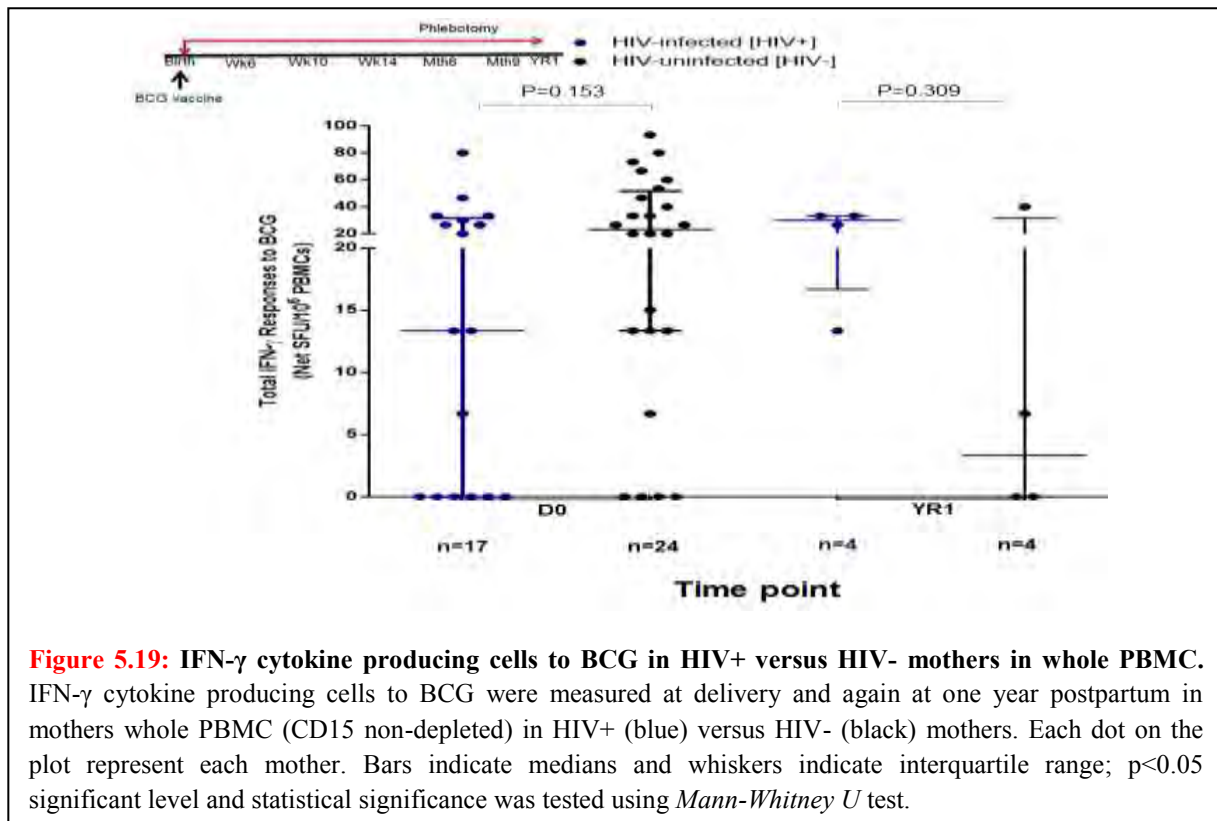
To further investigate if HIV-exposure could contribute to the effect of MDSC on IFN- γ cytokine producing cells, we evaluated IFN- γ production to BCG after MDSC depletion between HEU and HU infants at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age. At all time points, there were no significant differences at the individual time points in cells producing IFN- γ between groups (however, at one year, there were insufficient samples to be



measured in the HEU group and statistical analysis could not be performed; data not shown). As shown in **Figure 5.18**, in the grouped time point analysis there were no significant differences in IFN- γ producing cells to BCG at 6, 10 and 14 weeks grouped time points in HEU compared to HU infants (median of CD15 non-depleted=51.667 Net SFU/ 10^6 PBMC versus CD15 depleted=40.000 Net SFU/ 10^6 PBMC; $p=0.958$), as well as at 6, 9 months and one year grouped time points (median of CD15 non-depleted=36.330 Net SFU/ 10^6 PBMC versus CD15 depleted=18.000 Net SFU/ 10^6 PBMC; $p=0.301$) (**Figure 5.18**).

5.3.2.5 BCG induced IFN- γ cytokine producing cells in HIV+ versus HIV- mothers

We evaluated if HIV infection in mothers would alter the effect of MDSC on IFN- γ cytokine producing cells. First, we evaluated cells producing IFN- γ to BCG in whole PBMC (CD15 non-depleted) between HIV+ versus HIV- mothers at delivery and at one year postpartum, since HIV is known to alter T cell responses. As shown in **Figure 5.19**, there were no significant differences in IFN- γ cytokine producing cells between groups at all time points.

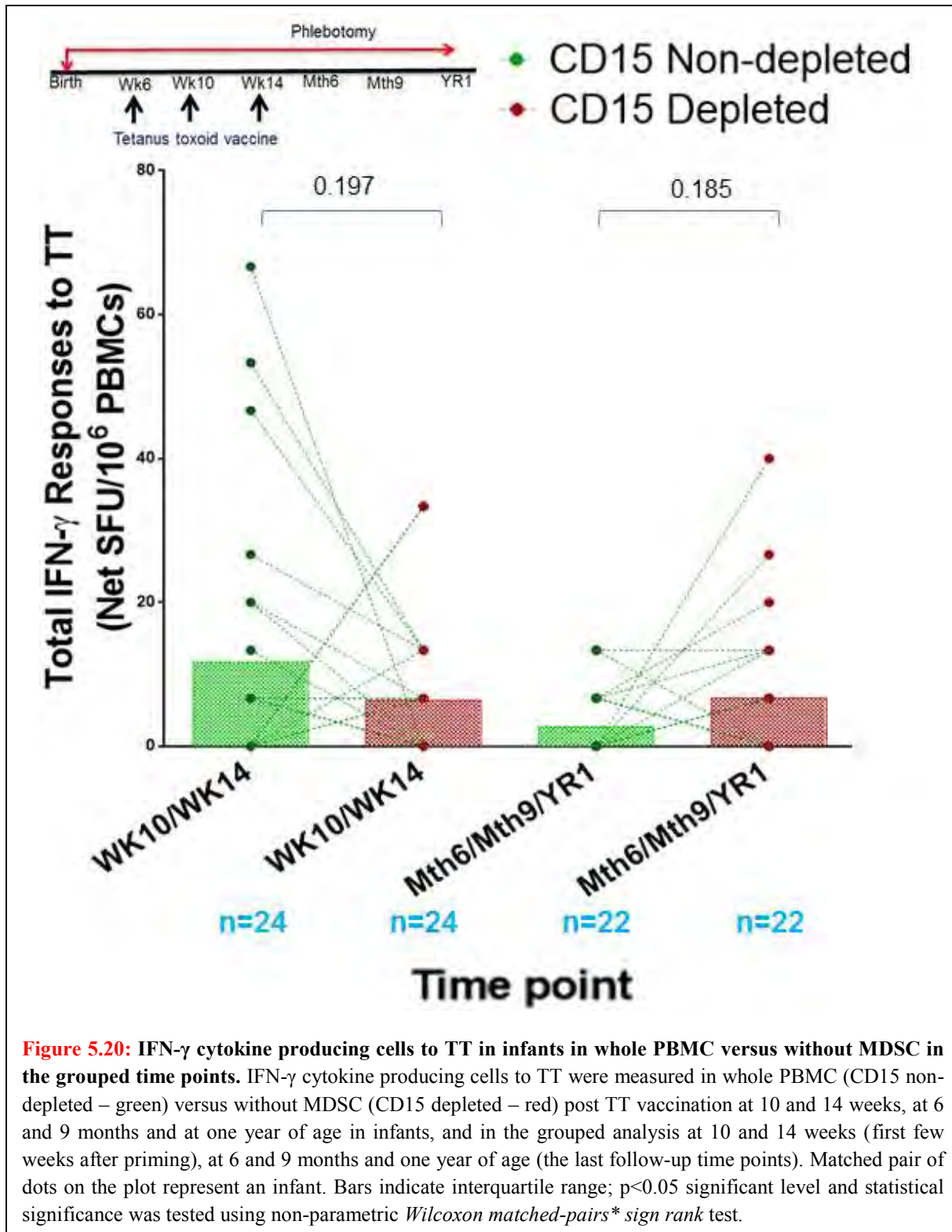


5.3.2.6 BCG induced IFN- γ cytokine producing cells in HIV+ versus HIV- mothers after MDSC depletion

Also, we evaluated cells producing IFN- γ to BCG in the absence of MDSC (CD15 depleted) between HIV+ versus HIV- mothers at delivery and at one year postpartum. There was no significant difference observed between groups at delivery (data not shown). However, at one year there were no HIV+ mothers for which this assay could be performed.

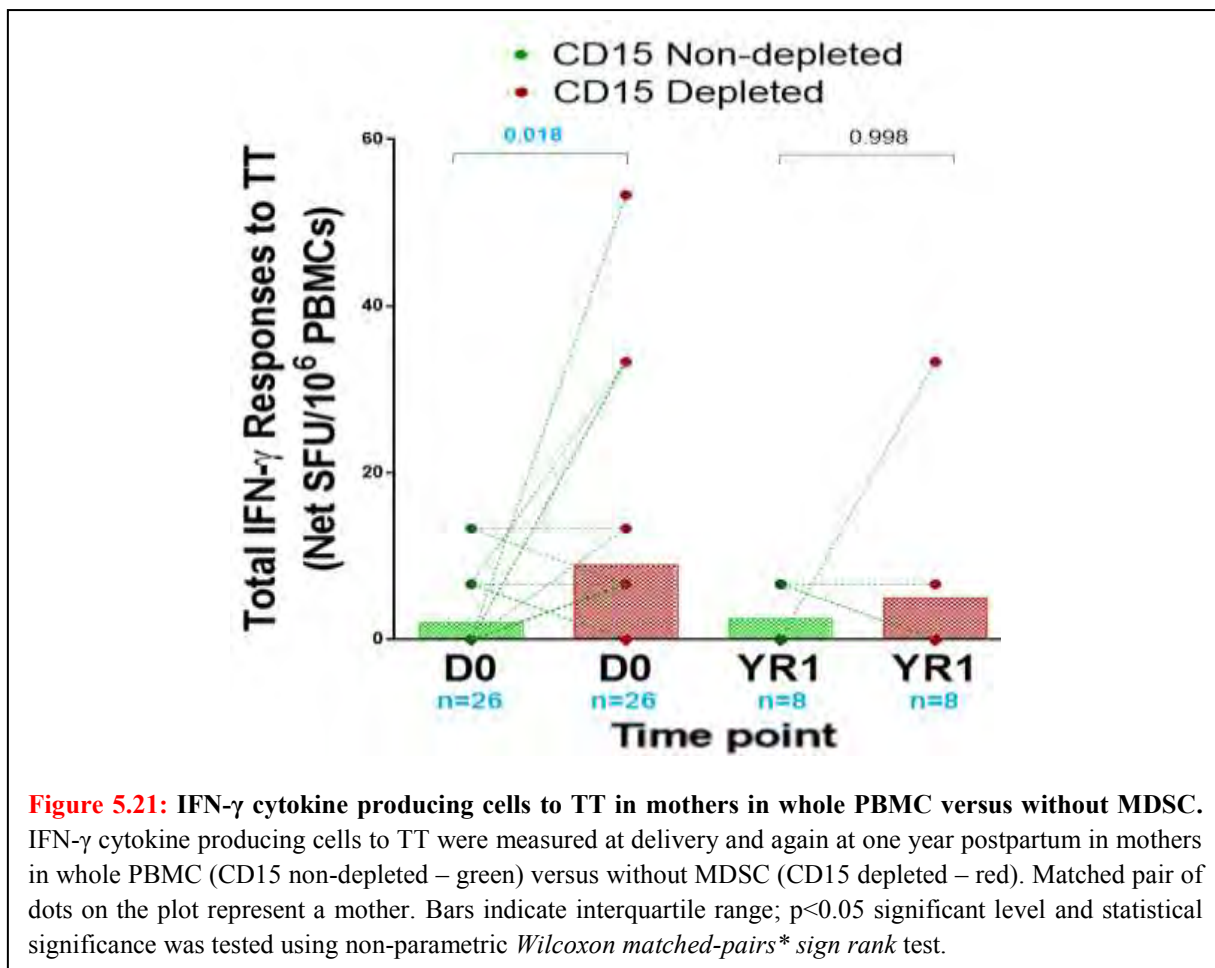
5.3.2.7 TT induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in infants

The cells producing IFN- γ to TT were evaluated in whole PBMC and without MDSC in infants post TT vaccination at 10 and 14 weeks, at 6 and 9 months, and at one year of age. There was no effect of MDSC depletion at any time point. However, IFN- γ producing cells to TT lasted till one year of age. There was no significant difference at the individual time points (however, at one year, there were not enough infants for which IFN- γ production could be measured and statistical analysis could not be performed; data not shown). In the grouped time point analysis there were no significant differences at 10 and 14 weeks (median of CD15 non-depleted=3.330 Net SFU/10⁶ PBMC versus CD15 depleted=0 Net SFU/10⁶ PBMC, $p=0.197$), and at 6, 9 months and one year (median of CD15 non-depleted=0 Net SFU/10⁶ PBMC, IQR=0-6.667, versus CD15 depleted=0 Net SFU/10⁶ PBMC, IQR=0-13.330; $p=0.185$) (Figure 5.20). Hence, there seems to be no effect of MDSC depletion of production of IFN- γ to TT in infants.



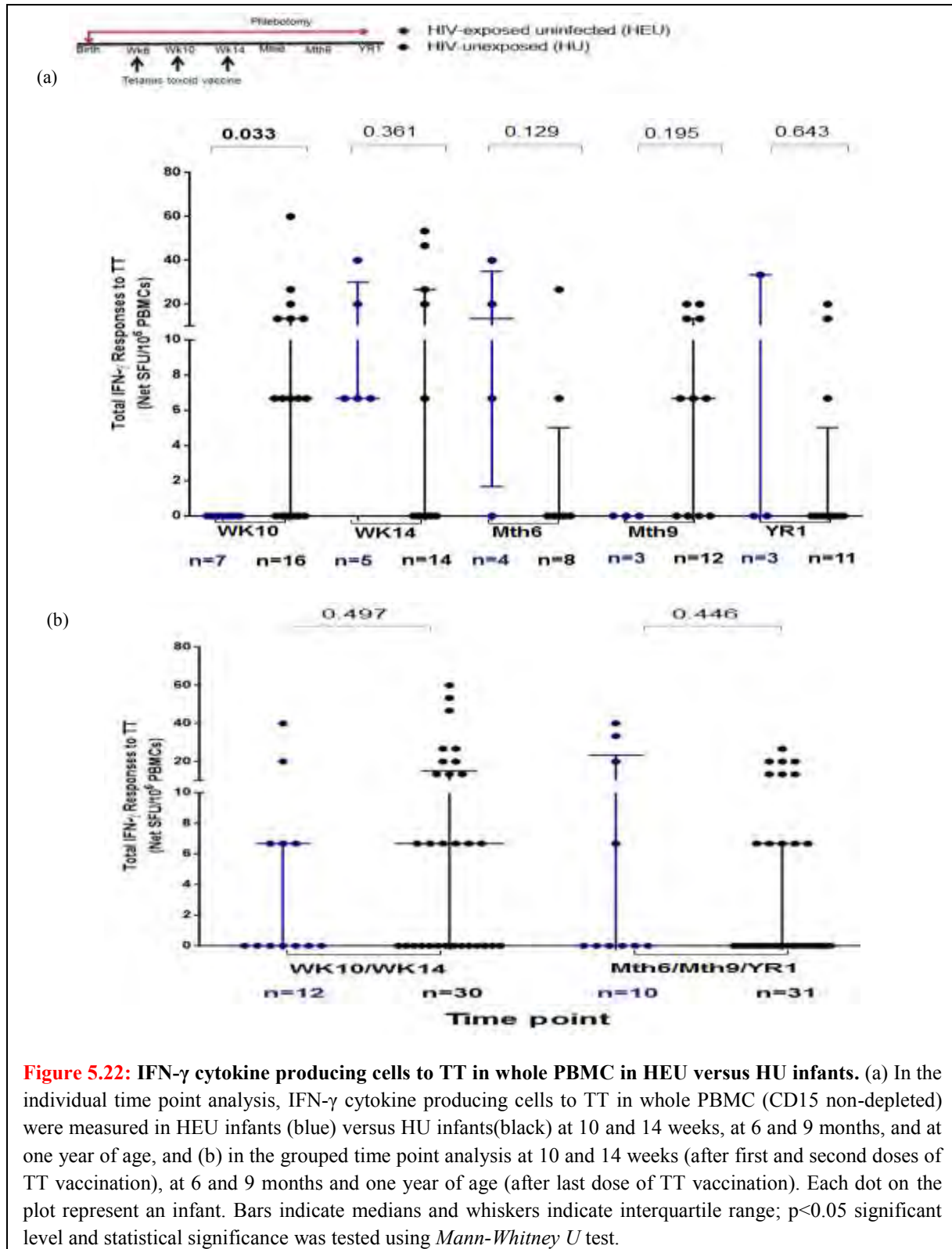
5.3.2.8 TT induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in mothers

In mothers, TT induced IFN- γ producing cells were evaluated in whole PBMC and without MDSC at delivery and again at one year postpartum. As shown in **Figure 5.21**, cells producing IFN- γ to TT were significantly lower before MDSC depletion in mothers at delivery (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC versus CD15 depleted=6.667 Net SFU/ 10^6 PBMC and $p=0.018$), but not at one year postpartum (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-6.667 versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-5.000 and $p=0.998$).



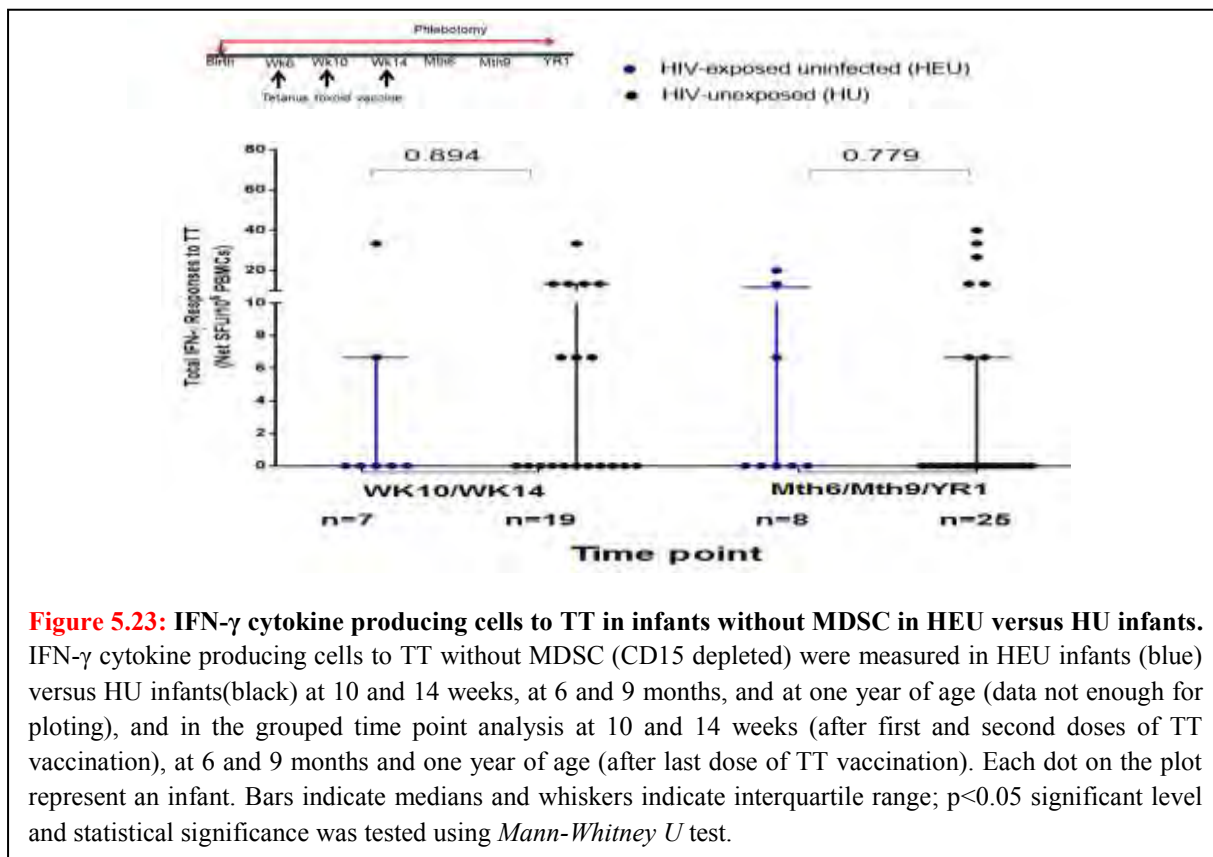
5.3.2.9 TT induced IFN- γ cytokine producing cells in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants

Next, we evaluated how HIV-exposure would contribute to the effect of MDSC on IFN- γ cytokine producing cells to TT. First we assessed whether HIV-exposure itself altered the cells producing IFN- γ to TT vaccination at 10 and 14 weeks, at 6 and 9 months and at one year of age. As shown in **Figure 5.22**, HIV-exposure significantly decreased IFN- γ responses at 10 weeks; cells producing IFN- γ to TT were significantly lower in HEU compared to HU infants (median of HEU=0 Net SFU/ 10^6 PBMC versus HU=6.667 Net SFU/ 10^6 PBMC and $p=0.033$). In the grouped time point analysis cells producing IFN- γ to TT were not significantly different between HEU compared to HU infants at at 10 and 14 weeks ($p=0.497$) as well as at 6, 9 months and one year ($p=0.446$).



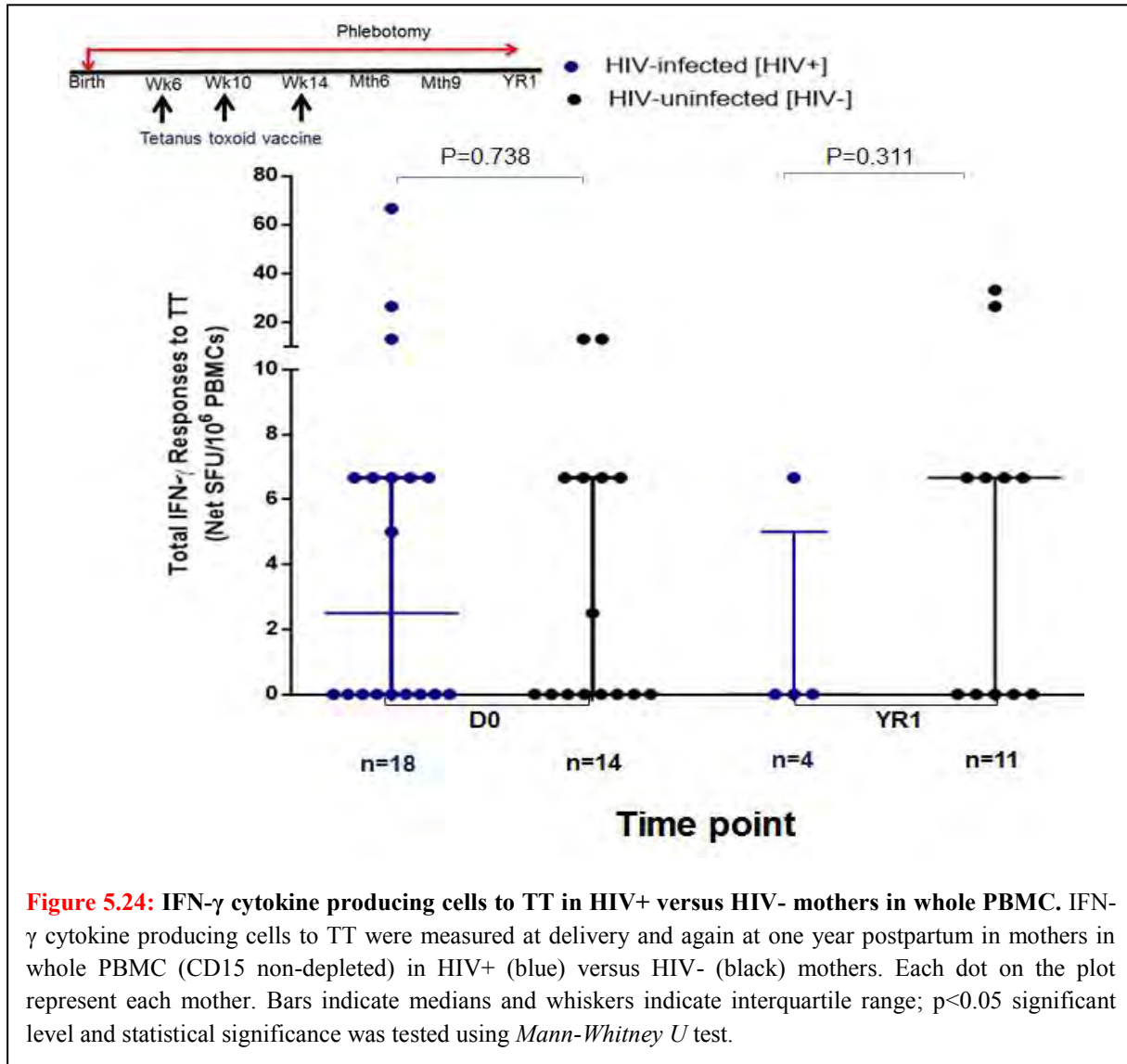
5.3.2.10 TT induced IFN- γ cytokine producing cells after MDSC depletion in HEU versus HU infants

To evaluate if HIV-exposure would alter the effect of MDSC on IFN- γ cytokine producing cells, we assessed cells producing IFN- γ to TT vaccination in the absence of MDSC between HEU versus HU infants at 10 and 14 weeks, at 6 and 9 months and at one year of age. At the individual time points, there were no significant differences in cells producing IFN- γ (however, at one year, there were no HEU infants for which IFN- γ cytokine producing cells to TT were measured and statistical analysis could not be performed; data not shown). As shown in **Figure 5.23**, in the grouped time point analysis there were no significant differences in cells producing IFN- γ to TT between HEU versus HU infants, at 10 and 14 weeks (median of HEU=0 Net SFU/ 10^6 PBMC, IQR=0-6.667 versus HU=0 Net SFU/ 10^6 PBMC, IQR=0-13.330; $p=0.894$), as well as at 6, 9 months and one year (median of HEU=0 Net SFU/ 10^6 PBMC, IQR=0-11.670 versus HU=0 Net SFU/ 10^6 PBMC, IQR=0-6.667; $p=0.779$).



5.3.2.11 TT induced IFN- γ cytokine producing cells in HIV+ versus HIV- mothers

We evaluated if HIV infection in mothers would alter IFN- γ cytokine producing cells to antigens. We assessed cells producing IFN- γ to TT in undepleted samples between HIV+ versus HIV- mothers at delivery and at one year postpartum. As shown in Figure 5.24, there were no significant differences observed at all time points.



5.3.2.12 TT induced IFN- γ cytokine producing cells in HIV+ versus HIV- mothers after MDSC depletion

We also evaluated if IFN- γ cytokine production would change after MDSC depletion in mothers to assess whether MDSC were functionally different in HIV infection. We assessed cells producing IFN- γ to TT in the absence of MDSC (CD15 depleted) between HIV+ versus HIV- mothers at delivery and again at one year postpartum. There was no significant difference observed between groups at birth. However, at one year, the number of HIV+ mothers for which cells producing IFN- γ to TT were to be measured was small and statistical analysis could not be performed (data not shown).

5.3.2.13 BP induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in infants

The cells producing IFN- γ to BP vaccination were evaluated in whole PBMC and without MDSC in infants post acellular Pertussis vaccination at 10 and 14 weeks, at 6 and 9 months, and at one year of age. There was no significant difference at the individual time points (however, at 6 months and one year of age, there were not enough infants for which IFN- γ cytokine producing cells to BP were measured and statistical analysis could not be performed; data not shown). In the grouped time point analyses, the differences were not significant after grouping infants at 10 and 14 weeks (median of CD15 non-depleted=6.667 Net SFU/ 10^6 PBMC, IQR=0-6.677, versus CD15 depleted=6.667 Net SFU/ 10^6 PBMC, IQR=0-10.000; $p=0.779$), and at 6, 9 months and one year (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-13.330, versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-10.000; $p=0.439$) (Figure 5.25). Hence, no major differences in cells producing IFN- γ before and after MDSC depletion at all time points was observed in infants.

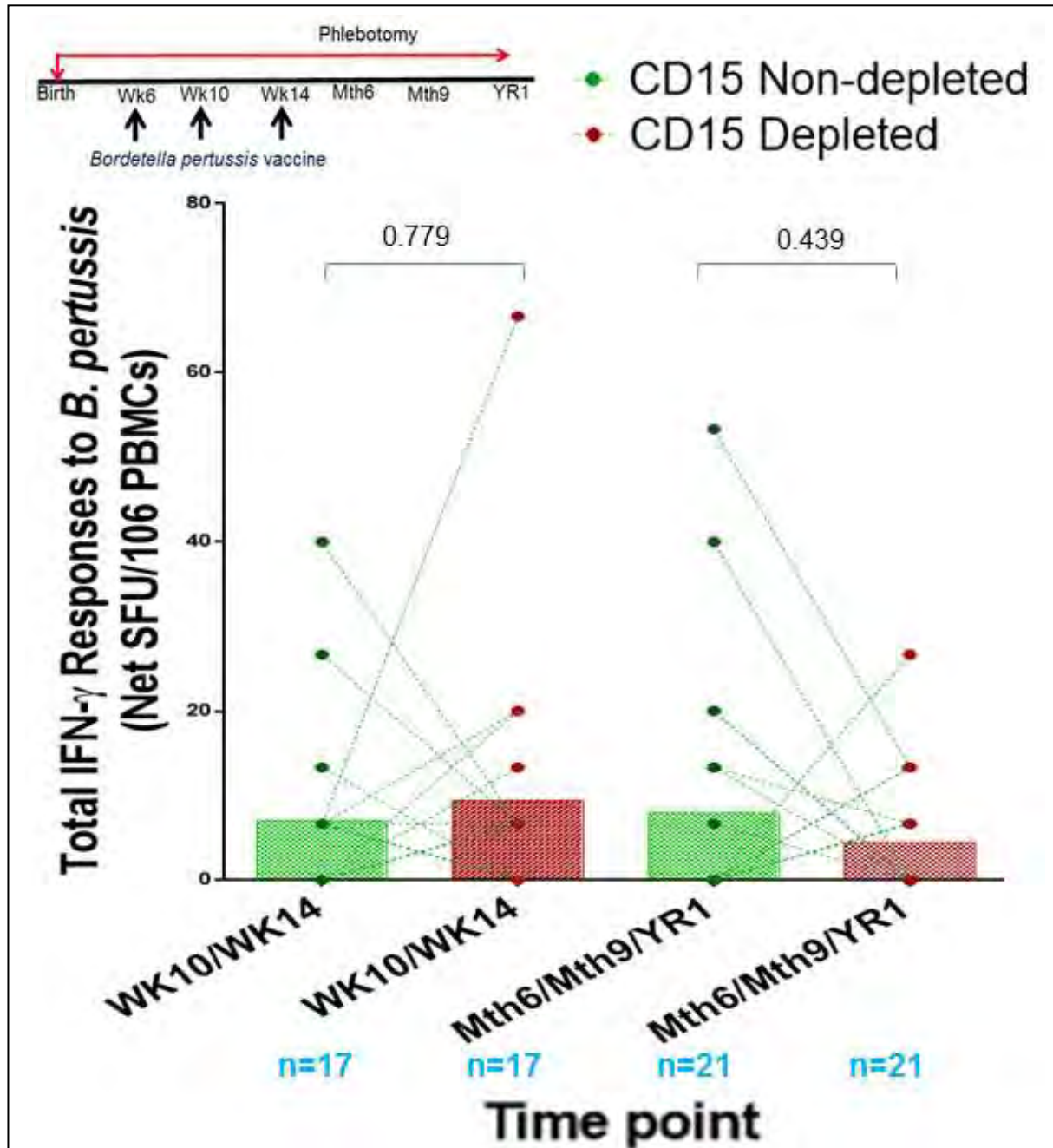
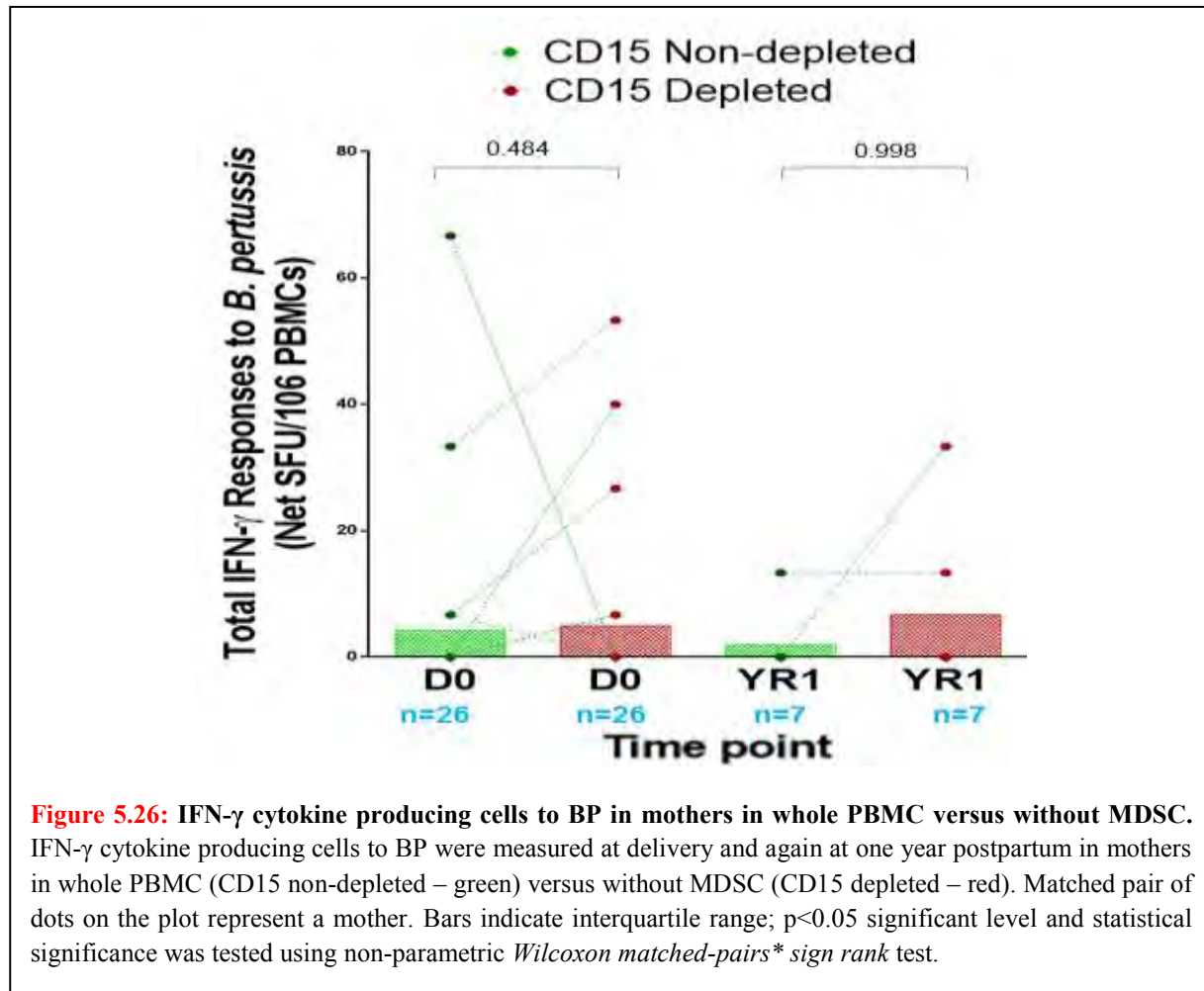


Figure 5.25: IFN- γ cytokine producing cells to BP in infants in whole PBMC versus without MDSC in the grouped time points. IFN- γ cytokine producing cells to BP were measured in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red) post BP vaccination at 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and in the grouped analysis at 10 and 14 weeks (first few weeks after priming), at 6 and 9 months and one year of age (the last follow-up time points). Matched pair of dots on the plot represent an infant. Bars indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using non-parametric *Wilcoxon matched-pairs* sign rank* test.

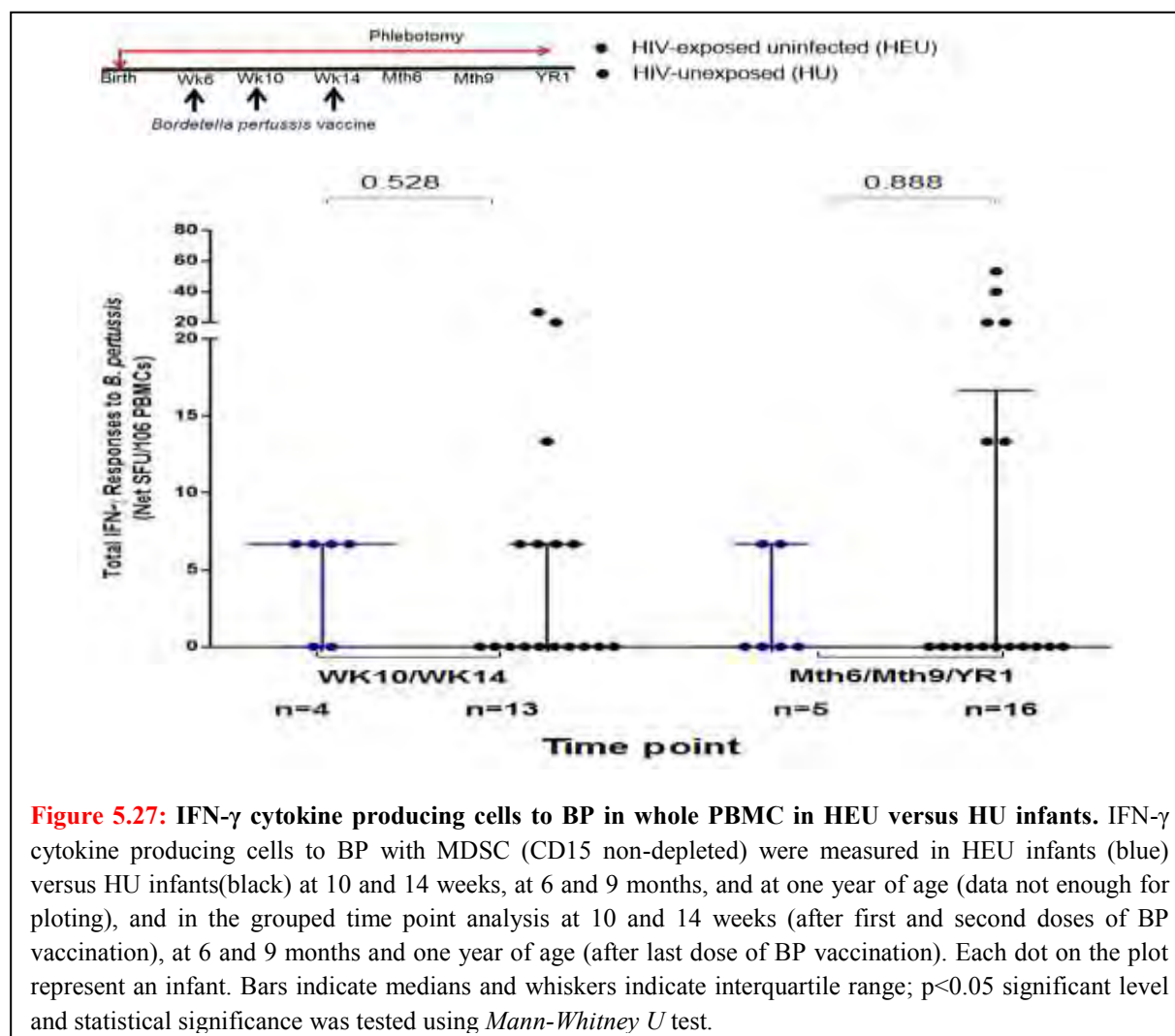
5.3.2.14 BP induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in mothers

In mothers, number of BP induced IFN- γ producing cells were evaluated in whole PBMC and without MDSC at delivery and again at one year postpartum. As shown in Figure 5.26, MDSC depletion did not affect IFN- γ production in mothers at either time point at delivery (median of CD15 non-depleted=0 Net SFU/10⁶ PBMC, IQR=0-0 versus CD15 depleted=0 Net SFU/10⁶ PBMC, IQR=0-0 and $p=0.484$), or at one year postpartum (median of CD15 non-depleted=0 Net SFU/10⁶ PBMC, IQR=0-0 versus CD15 depleted=0 Net SFU/10⁶ PBMC, IQR=0-13.330 and $p=0.998$). It is also important to note that, maternal IFN- γ producing cells to BP stimulation were very low, and most mothers did not respond to BP stimulation.



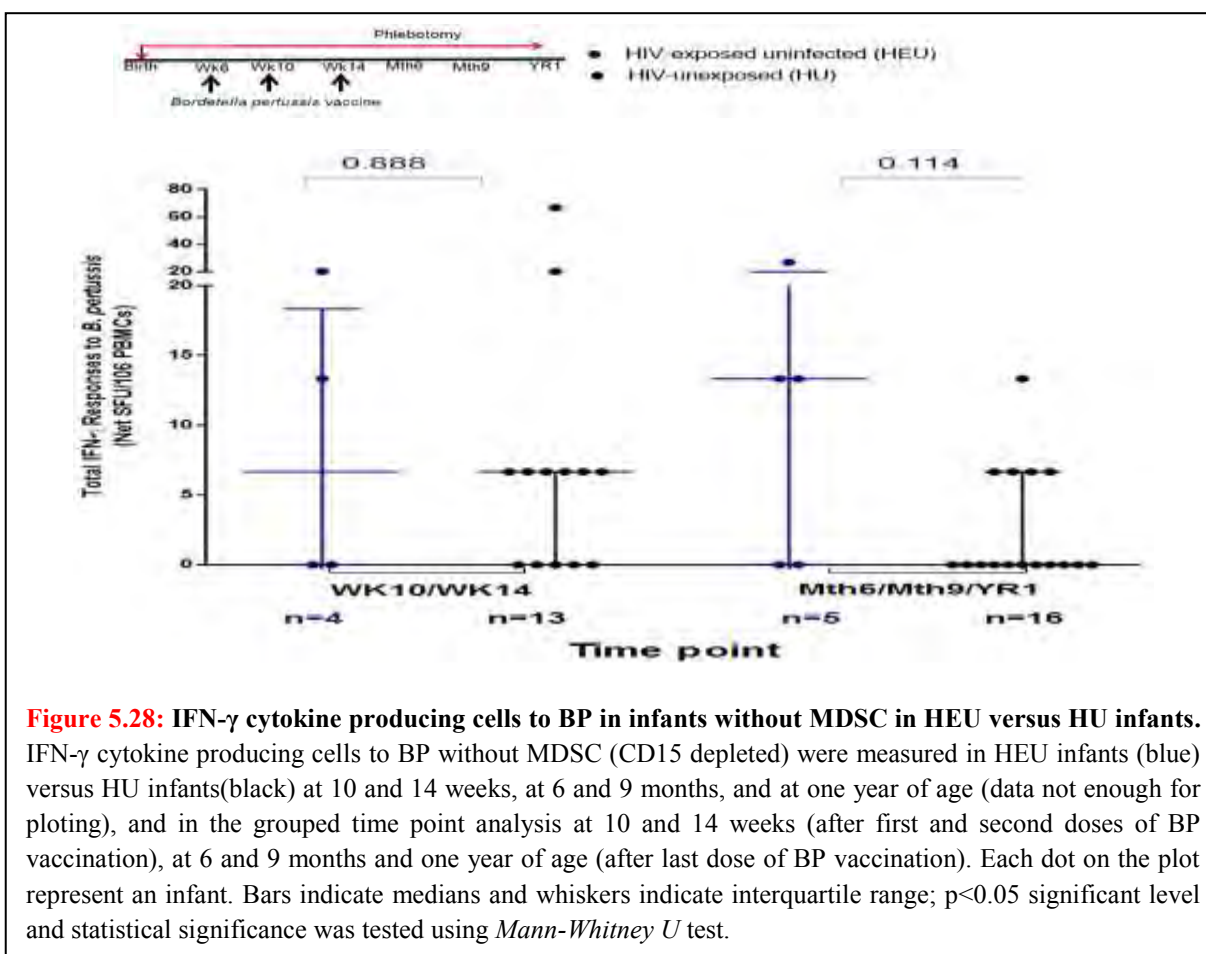
5.3.2.15 BP induced IFN- γ cytokine producing cells in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants

We evaluated cells producing IFN- γ to BP vaccination between HEU versus HU infants at 10 and 14 weeks, at 6 and 9 months and at one year of age. At the individual time points, there were no significant differences in cells producing IFN- γ (however, at one year, the number of HEU infants for which IFN- γ cytokine producing cells to BP was measured was small, and statistical analysis could not be performed; data not shown). In the grouped time point analysis as shown in **Figure 5.27**, there were no significant differences in cells producing IFN- γ to BP between HEU versus HU infants at all grouped time points.



5.3.2.16 BP induced IFN- γ production after MDSC depletion in HEU versus HU infants

To assess if HIV-exposure would contribute to the effect of MDSC on IFN- γ cytokine producing cells, we evaluated cells producing IFN- γ to BP vaccination in the absence of MDSC between HEU versus HU infants at 10 and 14 weeks, at 6 and 9 months and at one year of age. There were no significant differences at the individual time points or in grouped time points analyses in cells producing IFN- γ (however, at one year, the number of HEU infants for which IFN- γ cytokine producing cells to BP was measured was small, and statistical analysis could not be performed; data not shown). In the grouped time point analysis as shown in **Figure 5.28**, there were no significant differences in cells producing IFN- γ to BP between HEU versus HU infants at all grouped time points.



5.3.2.17 BP induced IFN- γ cytokine producing cells in HIV+ versus HIV- mothers

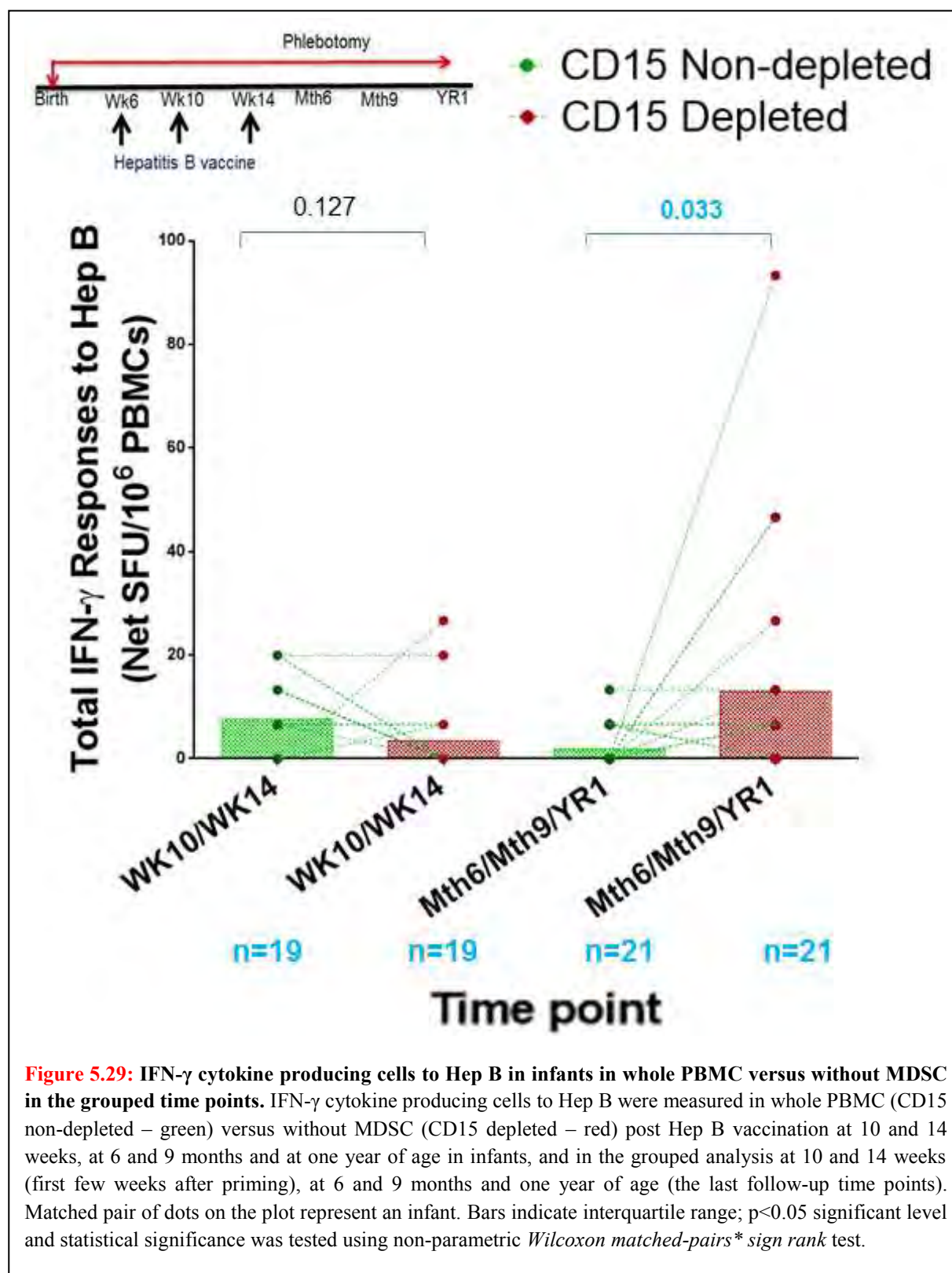
We assessed if HIV infection in mothers would alter IFN- γ cytokine producing cells. We evaluated cells producing IFN- γ to BP in the non-depleted samples between HIV+ versus HIV- mothers at delivery and again at one year postpartum. There were no significant differences observed in IFN- γ cytokine producing cells between groups at all time points. However, at one year, the number of HIV+ mothers for which cells producing IFN- γ to BP were measured was small and statistical analysis could not be performed (data not shown).

5.3.2.18 BP induced IFN- γ cytokine producing cells in HIV+ versus HIV- mothers after MDSC depletion

We assessed cell producing IFN- γ to BP in the absence of MDSC (CD15 depleted) between HIV+ versus HIV- mothers at delivery and at one year postpartum. There were no significant differences observed between groups at delivery. However, at one year, the number of HIV+ mothers for which cells producing IFN- γ to BP were measured was small and statistical analysis could not be performed (data not shown).

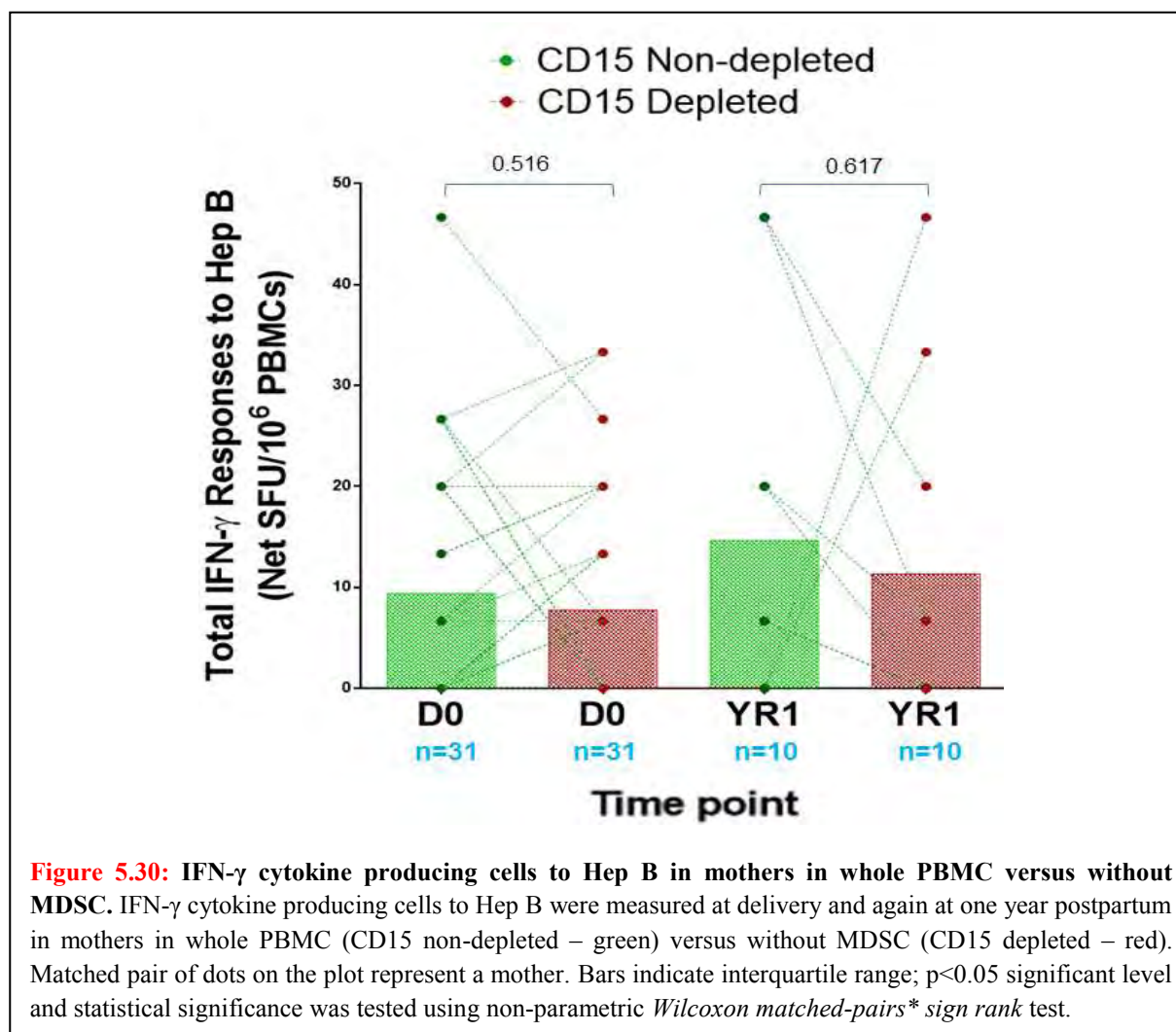
5.3.2.19 Hep B induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in infants

The cells producing IFN- γ to Hep B vaccine were evaluated in whole PBMC and without MDSC in infants at 10 and 14 weeks, at 6 and 9 months, and at one year of age. There was no significant difference at the individual time points (however, at 6 months and one year of age, there were not enough infants for which IFN- γ cytokine producing cells to BP were measured and statistical analysis could not be performed; data not shown). In the grouped time point analysis at 6, 9 months and one year grouped time points cells producing IFN- γ to Hep B vaccination stimulation were significantly higher after MDSC depletion than in whole PBMC (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-3.330 versus CD15 depleted=6.667 Net SFU/ 10^6 PBMC, IQR=0-13.330; $p=0.033$), but not at 10 and 14 weeks grouped together (median of CD15 non-depleted=6.667 Net SFU/ 10^6 PBMC versus CD15 depleted=0 Net SFU/ 10^6 PBMC; $p=0.127$) (Figure 5.29).



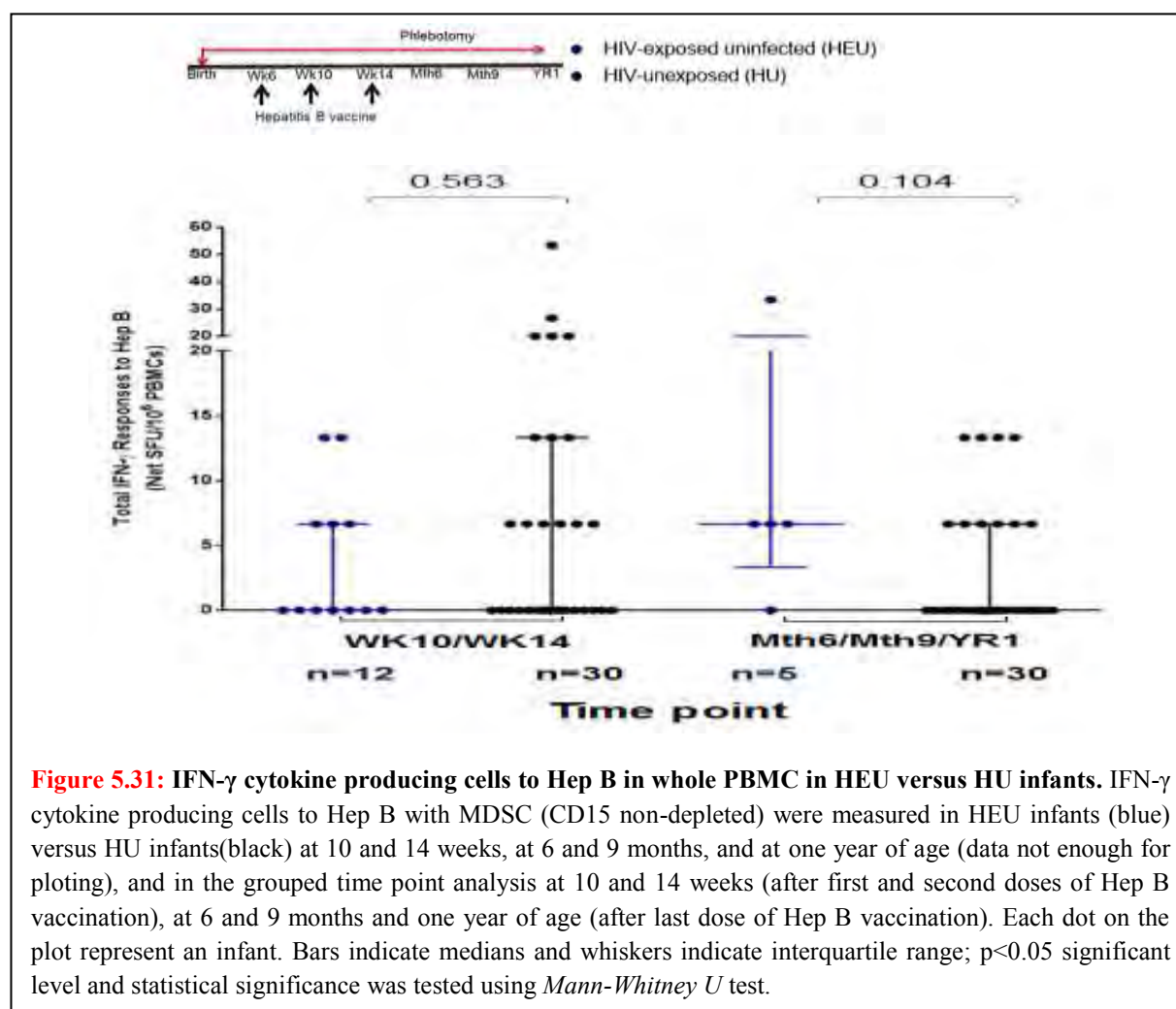
5.3.2.20 Hep B induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in mothers

In mothers, Hep B induced IFN- γ producing cells were evaluated with and without MDSC at delivery and at one year postpartum. As shown in Figure 5.30, cells producing IFN- γ to Hep B were not significantly different with and without MDSC in mothers after gestation at delivery (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-13.330 versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-3.330; $p=0.536$), and at one year postpartum (median of CD15 non-depleted=6.667 Net SFU/ 10^6 PBMC versus CD15 depleted=3.333 Net SFU/ 10^6 PBMC and $p=0.696$).



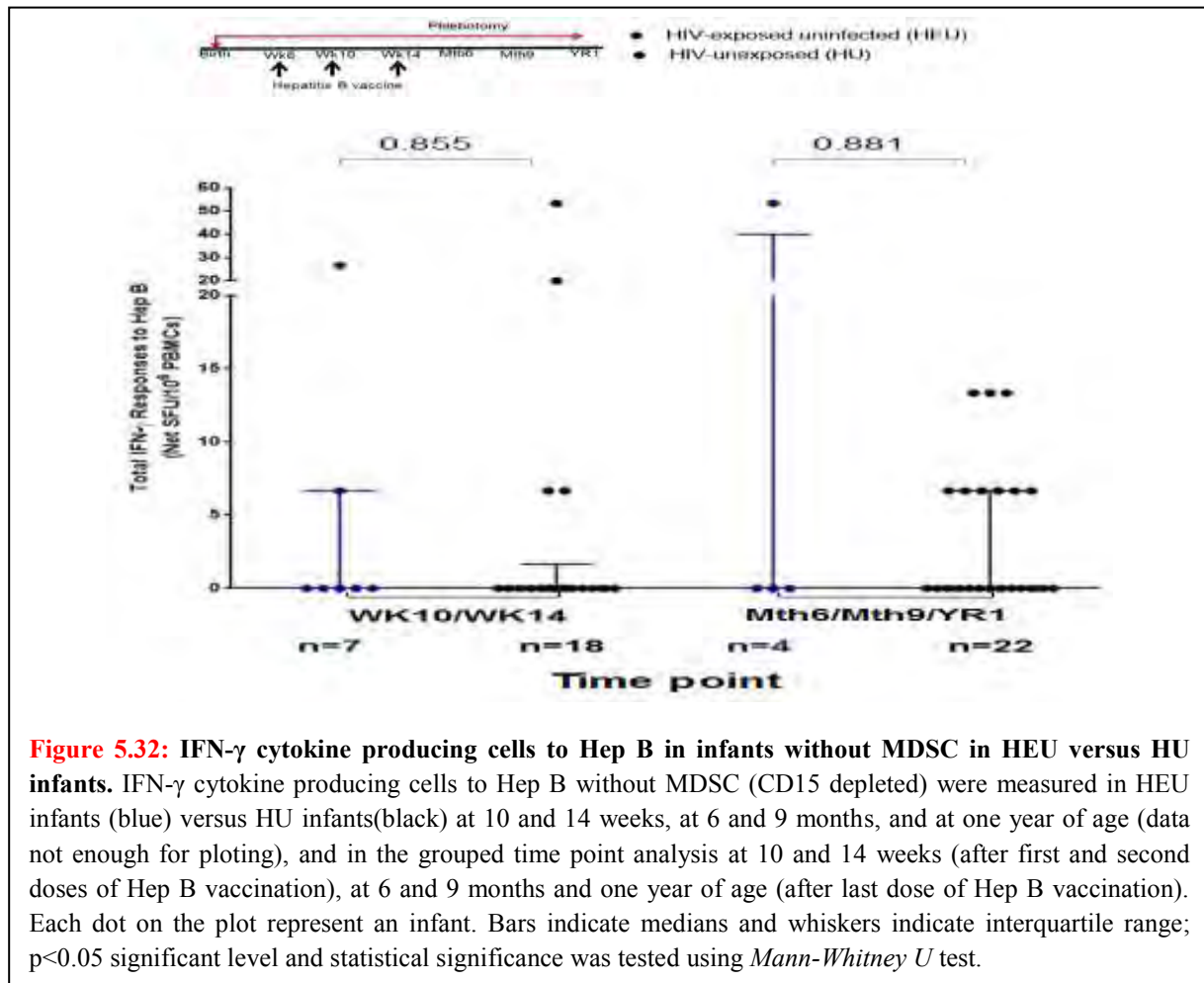
5.3.2.21 Hep B induced IFN- γ cytokine producing cells in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants

We evaluated cells producing IFN- γ to Hep B vaccination between HEU versus HU infants at 10 and 14 weeks, at 6 and 9 months and at one year of age. There were no significant differences at the individual time points in cells producing IFN- γ (however, at 9 months, there were no HEU infants for which IFN- γ cytokine producing cells to Hep B were measured, and statistical analysis could not be performed; data not shown). In the grouped time point analysis as shown in **Figure 5.31**, there were no significant differences in cells producing IFN- γ to Hep B between HEU versus HU infants.



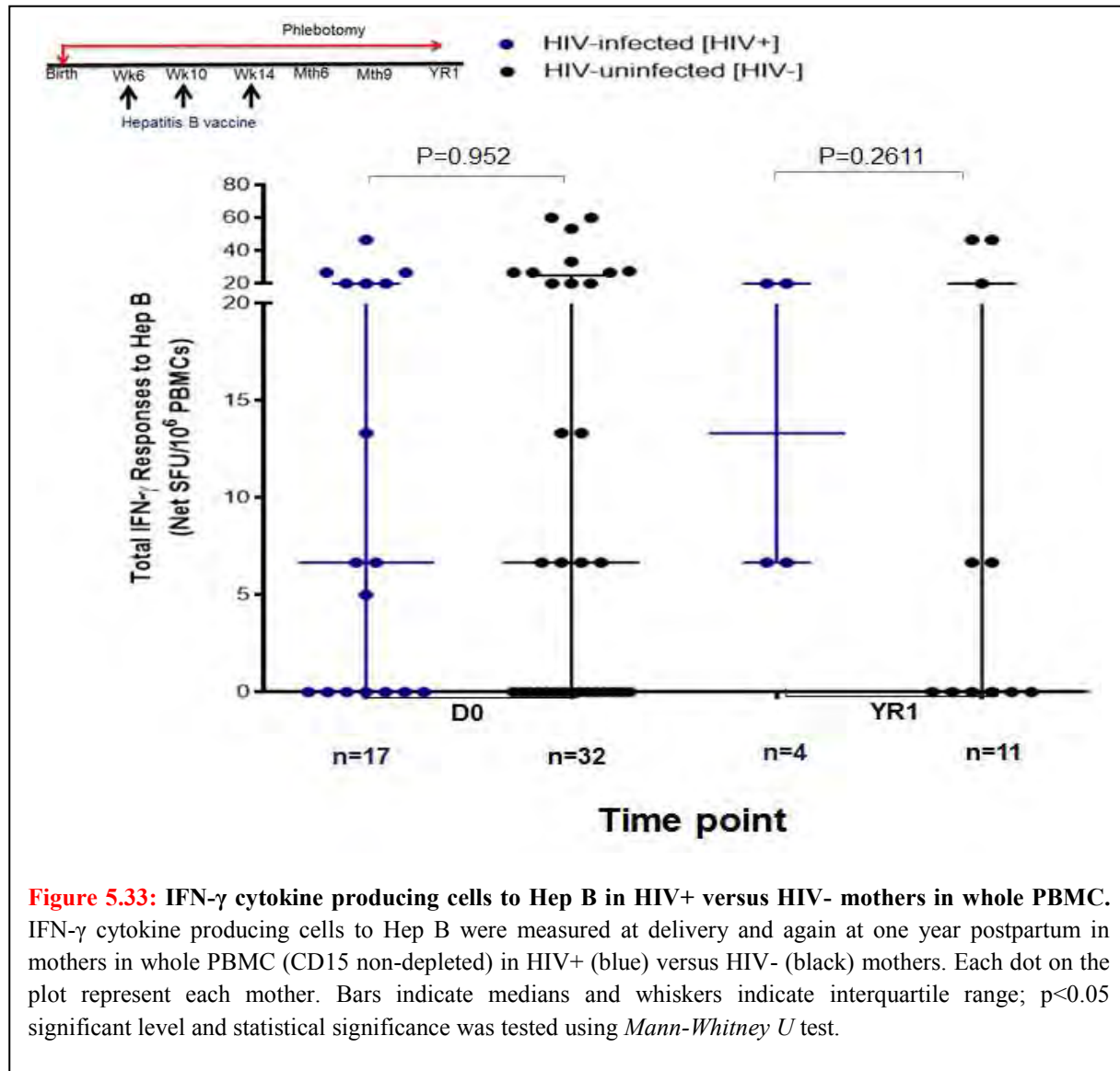
5.3.2.22 Hep B induced IFN- γ cytokine producing cells after MDSC depletion in HEU versus HU infants

We evaluated cells producing IFN- γ to Hep B vaccination in the absence of MDSC between HEU versus HU infants at 10 and 14 weeks, at 6 and 9 months and at one year of age. At the individual time points, there were no significant differences at most time points in cells producing IFN- γ (however, at 6 weeks the number of HEU infants was small, and at 9 months and one year of age, there were no HEU infants for which IFN- γ cytokine producing cells to Hep B were measured, and statistical analysis could not be performed; data not shown). In the grouped time point analysis as shown in [Figure 5.32](#), there were no significant differences in cells producing IFN- γ to Hep B between HEU versus HU infants at all grouped time points.



5.3.2.23 Hep B induced IFN- γ cytokine producing cells in HIV+ versus HIV- mothers

We assessed cell producing IFN- γ to Hep B in non-depleted samples from HIV+ versus HIV- mothers at delivery and at one year postpartum. There were no significant differences in IFN- γ cytokine producing cells at either time point (Figure 5.33).



5.3.2.24 Hep B induced IFN- γ cytokine producing cells in HIV+ versus HIV- mothers after MDSC depletion

We evaluated cell producing IFN- γ to Hep B in the absence of MDSC (CD15 depleted) between HIV+ versus HIV- mothers at delivery and at one year postpartum. There were no significant differences IFN- γ cytokine producing cells between groups at all time points. However, at one year, the number of HIV+ mothers for which cells producing IFN- γ to Hep B were measured was small and statistical analysis could not be performed (data not shown).

5.3.2.25 PHA induced IFN- γ cytokine producing cells in the presence of MDSC versus after MDSC depletion in infants

Phytohaemagglutinin (PHA) has been widely described and its use as a mitogen to trigger T lymphocyte cell proliferation and IFN- γ production as well as other cytokines (Mire-Sluis *et al.*, 1987) (Gondo-Rey *et al.*, 2001). We employed PHA as a positive control and we also assessed how MDSC would affect cells producing IFN- γ to PHA. We evaluated IFN- γ cytokine producing cells to PHA in whole PBMC and without MDSC in infants at birth, at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age (Figure 5.34). IFN- γ cytokine

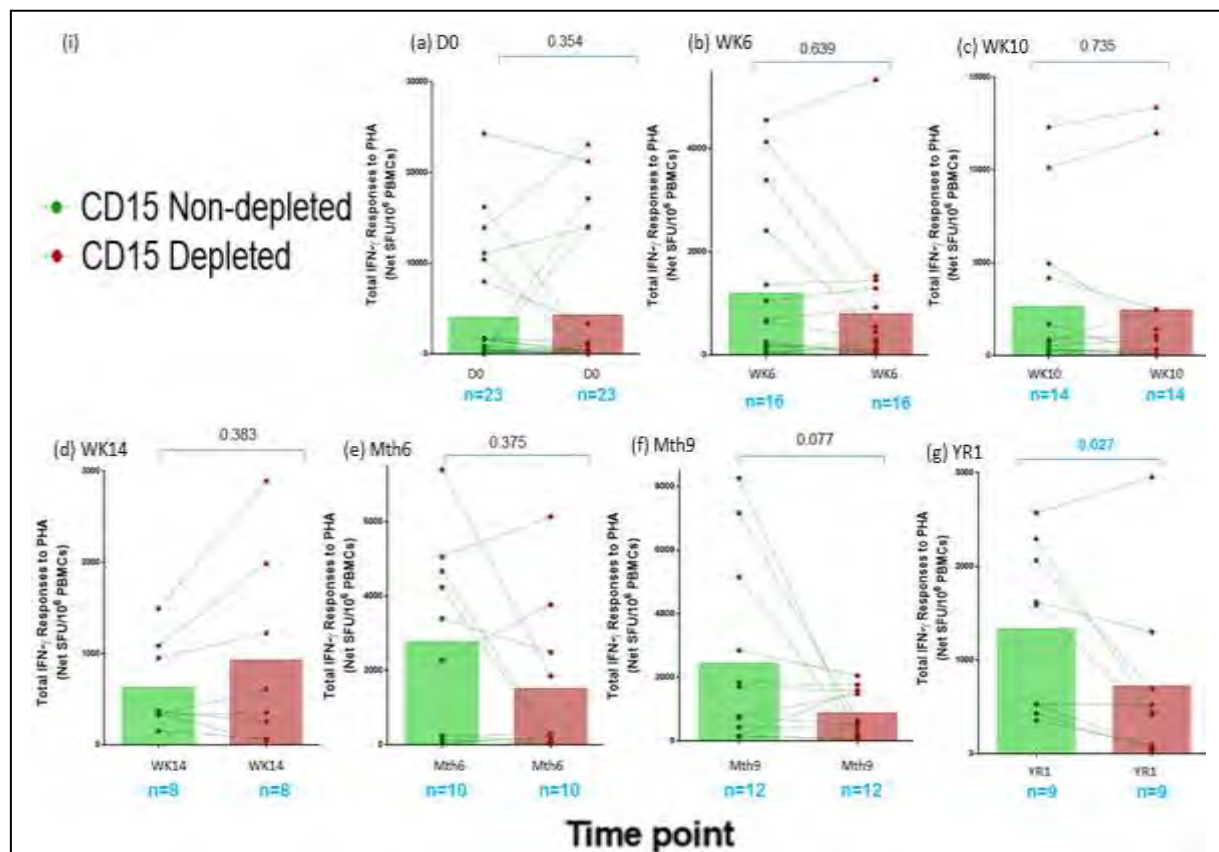


Figure 5.34 (i): IFN- γ cytokine producing cells to PHA in infants in whole PBMC versus without MDSC in the individual time points. In the individual time point analyses, IFN- γ cytokine producing cells to PHA were measured in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red) at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants. Matched pair of dots on the plot represent an infant. Bars indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using non-parametric Wilcoxon matched-pairs* sign rank test.

producing cells to PHA lasted till one year and responses were robust at all time points. IFN- γ cytokine producing cells to PHA were a significantly lower at one year of age in infants after MDSC depletion than in whole PBMC (median of CD15 non-depleted=1587.000 Net SFU/ 10^6 PBMC versus CD15 depleted=446.700 Net SFU/ 10^6 PBMC and $p=0.027$) [Figure 5.34 (i) (g)]. There were no significant differences observed at other time points at birth ($p=0.354$), at 6, 10 and 14 weeks ($p=0.639$, $p=0.735$ and $p=0.383$ respectively), and at 6 and 9 months ($p=0.375$ and $p=0.077$ respectively). Also, infants were grouped at birth and 6 weeks, at 10 and 14 weeks, and at 6 and 9 months and one year of age grouped time points. The difference observed at one year of age at the individual time point was evident at the 6 and 9 months and one year grouped time point. IFN- γ cytokine producing cells to PHA were a significantly lower at 6 and 9 months and one year grouped time points in infants after MDSC depletion than in whole PBMC (median of CD15 non-depleted=1620.000 Net SFU/ 10^6 PBMC versus CD15 depleted=640.000 Net SFU/ 10^6 PBMC and $p=0.007$) [Figure 5.34 (ii)]. There were no significant differences at birth and 6 weeks as well as at 10 and 14 weeks grouped time points ($p=0.439$ and $p=0.406$ respectively).

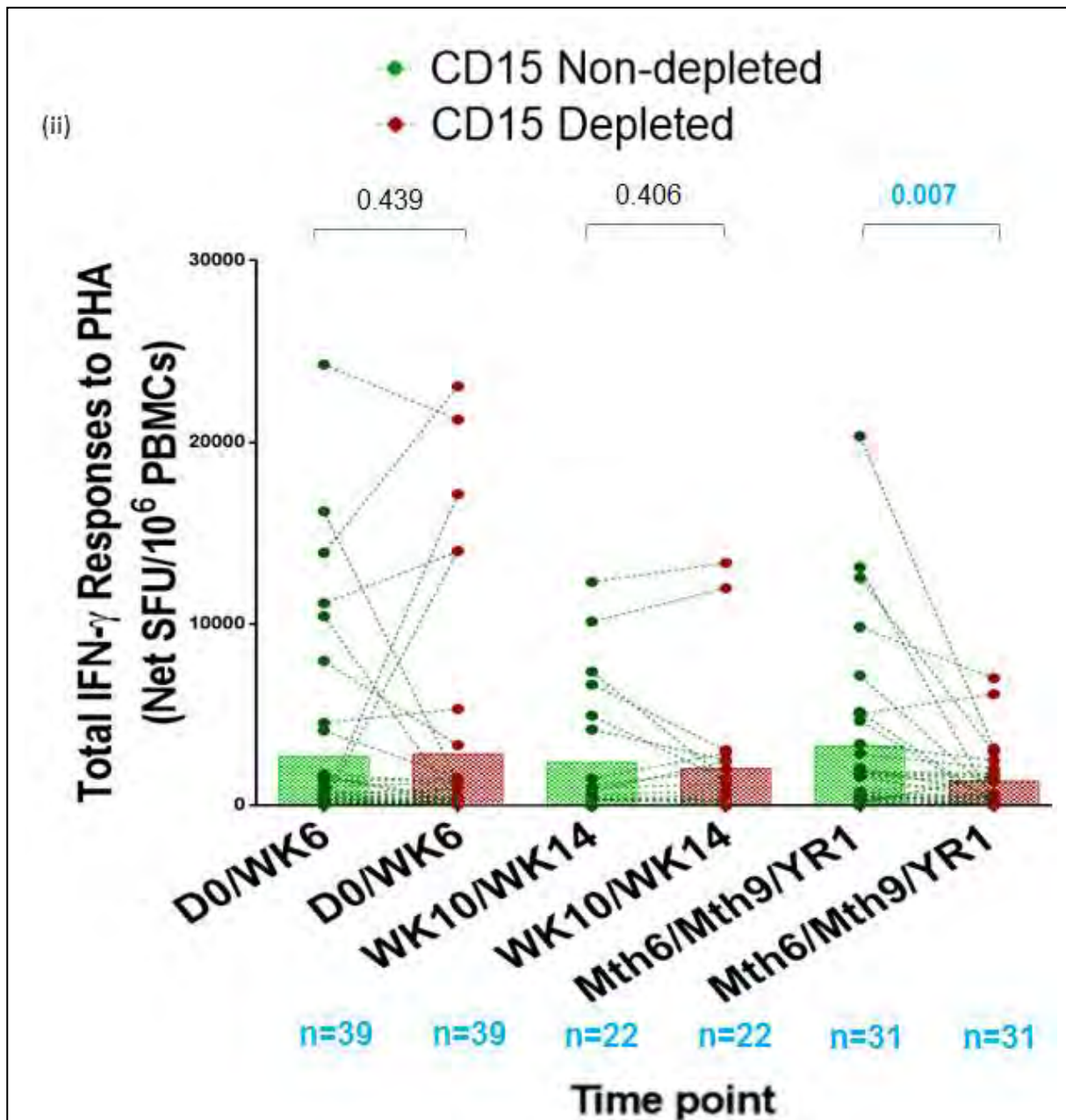
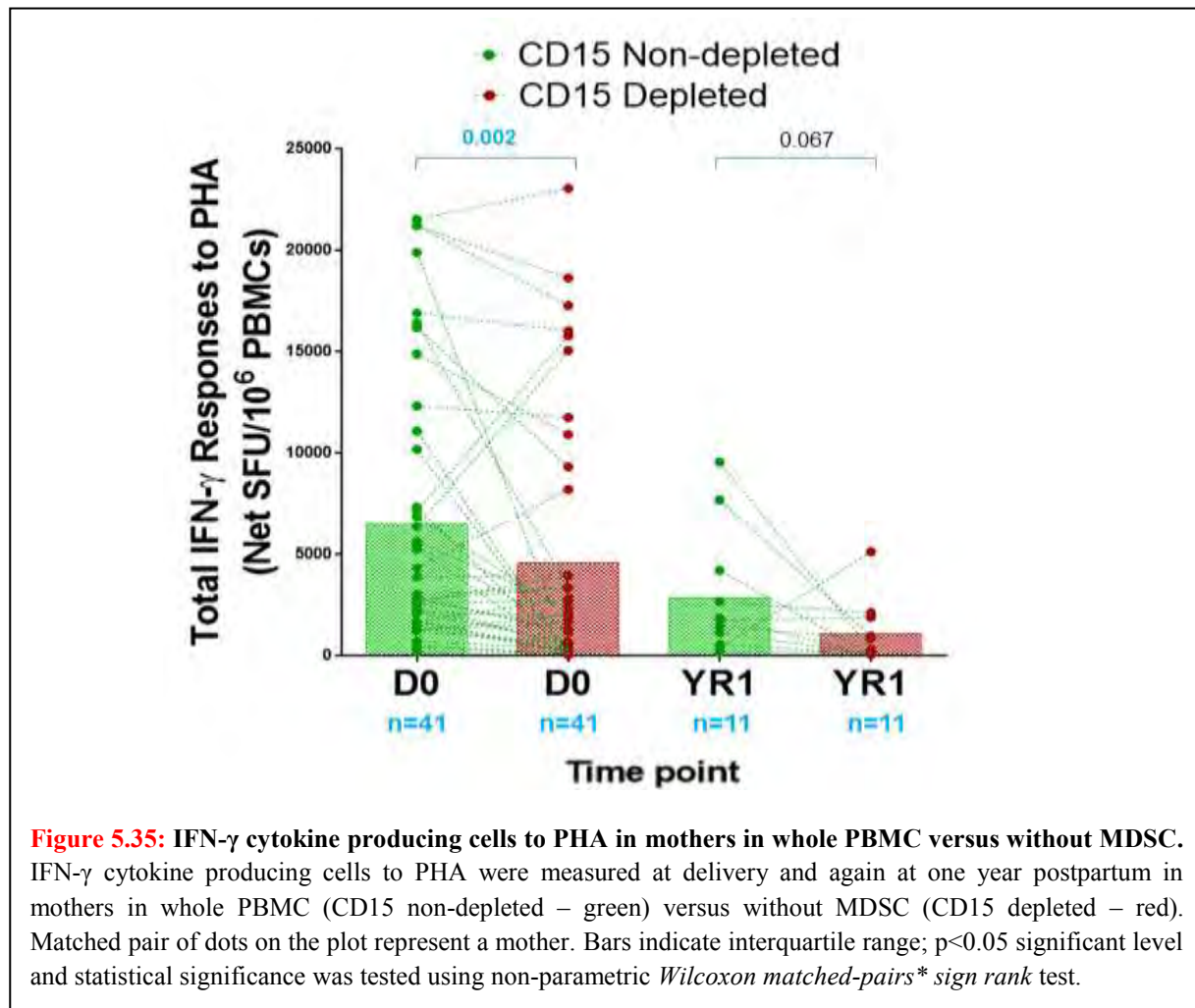


Figure 5.34 (ii): IFN- γ cytokine producing cells to PHA in infants in whole PBMC versus without MDSC in the grouped time points. IFN- γ cytokine producing cells to PHA were measured in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red) at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and in the grouped analysis at 6, 10 and 14 weeks (first few weeks after priming), at 6 and 9 months and one year of age (the last follow-up time points). Matched pair of dots on the plot represent an infant. Bars indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using non-parametric *Wilcoxon matched-pairs* sign rank* test.

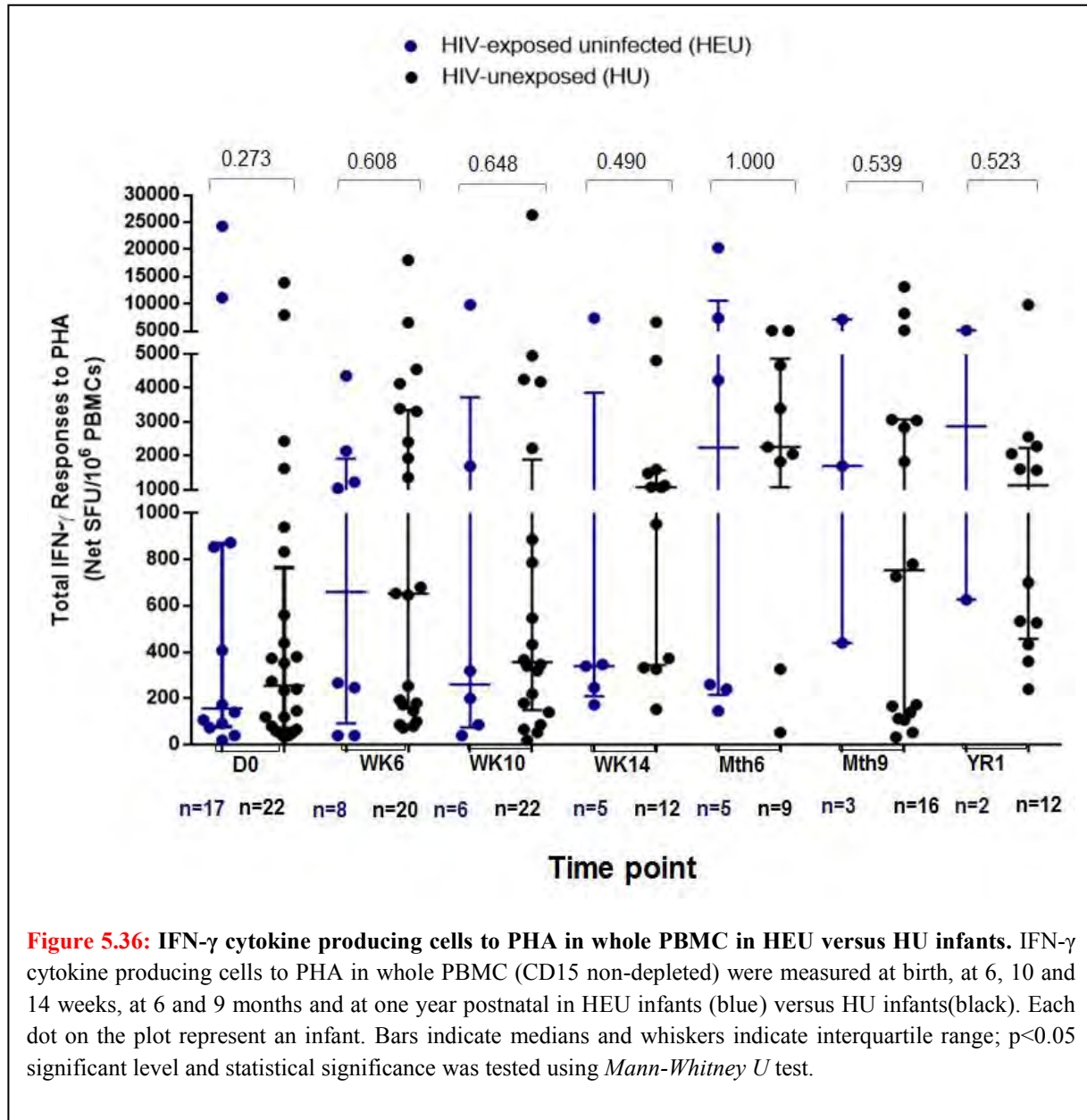
5.3.2.26 PHA induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in mothers

In mothers, PHA induced IFN- γ producing cells were compared in whole PBMC and without MDSC at delivery and at one year postpartum. As shown in [Figure 5.35](#), cells producing IFN- γ to PHA were significantly higher in whole PBMC than after MDSC depletion in mothers at delivery (median of CD15 non-depleted=3047.000 Net SFU/10⁶ PBMC versus CD15 depleted=1947.000 Net SFU/10⁶ PBMC and $p=0.002$), but not at one year postpartum (median of CD15 non-depleted=1773.000 Net SFU/10⁶ PBMC versus CD15 depleted=320.000 Net SFU/10⁶ PBMC and $p=0.067$).



5.3.2.27 PHA induced IFN- γ cytokine producing cells in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants

We evaluated cells producing IFN- γ to PHA in the presence of MDSC between HEU versus HU infants at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age. As shown in **Figure 5.36**, there were no significant differences at any time point.



5.3.2.28 PHA induced IFN- γ cytokine producing cells after MDSC depletion in HEU versus HU infants

We assessed cells producing IFN- γ to PHA in the absence of MDSC between HEU versus HU infants at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age. At all the time points, there were no significant differences in cells producing IFN- γ between groups. However, at one year of age there were no HEU infants for which IFN- γ cytokine producing cells to PHA were measured (data not shown).

5.3.2.29 PHA induced IFN- γ cytokine producing cells in HIV+ versus HIV- mothers

We evaluated cell producing IFN- γ to PHA between HIV+ versus HIV- mothers at delivery and at one year postpartum. As shown in [Figure 5.37](#), there were no significant differences in IFN- γ cytokine producing cells between groups at either time point.

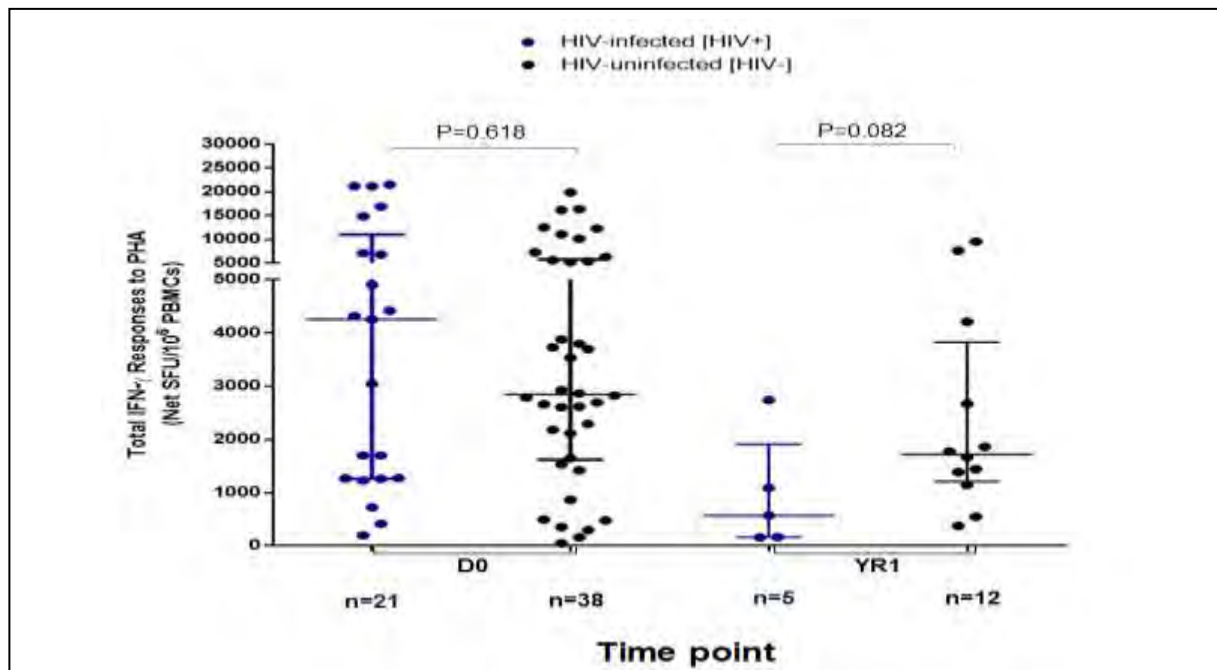


Figure 5.37: IFN- γ cytokine producing cells to PHA in HIV+ versus HIV- mothers in whole PBMC. IFN- γ cytokine producing cells to PHA were measured at delivery and again at one year postpartum in mothers in whole PBMC (CD15 non-depleted) in HIV+ (blue) versus HIV- (black) mothers. Each dot on the plot represent each mother. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

5.3.2.30 PHA induced IFN- γ cytokine producing cells in HIV+ versus HIV- mothers after MDSC depletion

We evaluated cell producing IFN- γ to PHA in the absence of MDSC (CD15 depleted) between HIV+ versus HIV- mothers at delivery and at one year postpartum. As shown in Figure 5.38, at delivery, HIV+ mothers had a significantly higher IFN- γ cytokine producing cells compared to their uninfected counterparts (median of CD15 non-depleted=3393.000 Net SFU/ 10^6 PBMC versus CD15 depleted=930.000 Net SFU/ 10^6 PBMC and $p=0.007$). By one year postpartum, this difference was no longer significant between groups (median of CD15 non-depleted=156.700 Net SFU/ 10^6 PBMC versus CD15 depleted=846.700 Net SFU/ 10^6 PBMC and $p=0.582$).

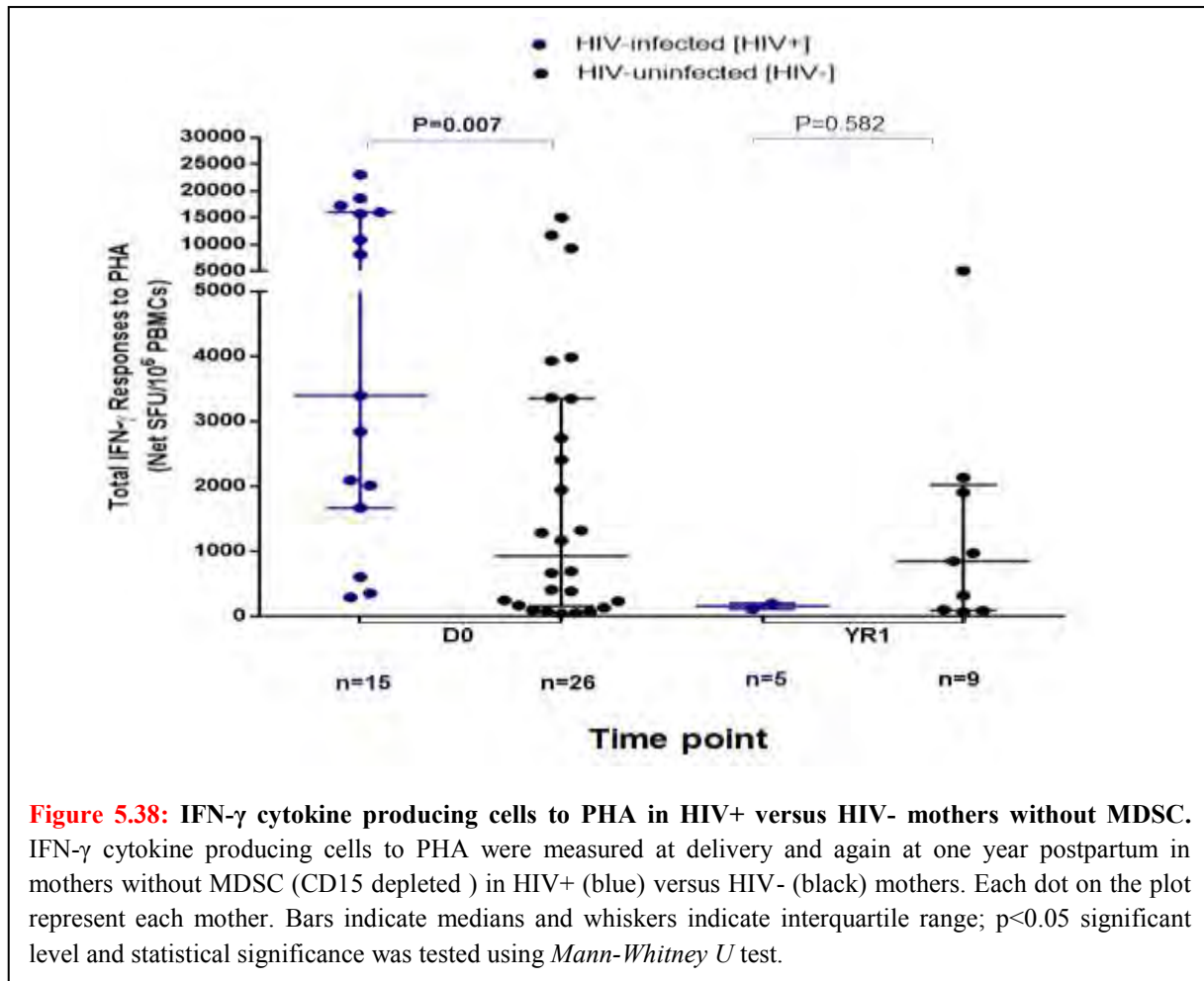


Table 5.4: Summary results of the quantity of IFN- γ cytokine producing cells in response to vaccines and antigens stimulations as measured in PBMC (presence of MDSC) versus without MDSC (after MDSC depletion) in infants and mothers. P-values of infants and mothers IFN- γ cytokine producing cells in PBMC (presence of MDSC) versus without MDSC (after MDSC depletion) after stimulation with the following antigens [Bacillus Calmette-Guérin (BCG), Hepatitis B (Hep B), Tetanus toxoid (TT), *Bordetella pertussis* (BP) and Phytohaemagglutinin (PHA)]. Blue highlights represent the parameters (p-values and type of data sets), Red highlights represent the different time points [birth (D0), 6, 10 and 14 weeks (WK6, WK10 and WK14), 6 and 9 months (Mth6 and Mth9), and 1 year (YR1)], and Purple highlights represent significant differences in IFN- γ cytokine producing cells in the presence of MDSC versus after MDSC depletion to vaccine antigens. GRP=Group, Indv=Individual, TP= Time point, B=Baby, and M=Mother.

Parameters	p-values						
Time points	D0	WK6	WK10	WK14	Mth6	Mth9	YR1
Hep B GRP TP (B)			0.127		0.033		
Hep B Indv.TP (M)	0.516						0.617
TT GRP TP (B)			0.197		0.185		
TT Indv.TP (M)	0.018						0.998
BCG Indv TP (B)		0.147	0.191	0.203	0.109	0.061	0.016
BCG GRP TP (B)		0.013			0.001		
BCG Indv.TP (M)	0.739						0.125
BP GRP TP (B)			0.779		0.439		
BP Indv.TP (M)	0.484						0.998
PHA Indv TP (B)	0.354	0.639	0.735	0.383	0.375	0.077	0.027
PHA GRP TP (B)	0.439		0.406		0.007		
PHA Indv.TP (M)	0.002						0.067

Table 5.5: Summary results of the quantity of IFN- γ cytokine producing cells in response to vaccines and antigens stimulations as measured in HEU versus HU infants as well as in HIV+ versus HIV- mothers in PBMC (presence of MDSC) and without MDSC (after MDSC depletion). P-values of infants and mothers IFN- γ cytokine producing cells in PBMC (presence of MDSC) and without MDSC (after MDSC depletion) in HEU versus HU infants as well as in HIV+ versus HIV- mothers after stimulation with the following antigens [Bacillus Calmette-Guérin (BCG), Hepatitis B (Hep B), Tetanus toxoid (TT), *Bordetella pertussis* (BP) and Phytohaemagglutinin (PHA)]. Blue highlights represent the parameters (p-values and type of data sets), Red highlights represent the different time points [birth (D0), 6, 10 and 14 weeks (WK6, WK10 and WK14), 6 and 9 months (Mth6 and Mth9), and 1 year (YR1)], and Purple highlights represent significant differences in IFN- γ cytokine producing cells to vaccine antigens in the presence and absence of MDSC in HEU versus HU infants. GRP=Group, Indv=Individual, TP= Time point, B=Baby, M=Mother, N=MDSC non-depleted, and D=MDSC depleted.

Parameters	p-values						
Time points	D0	WK6	WK10	WK14	Mth6	Mth9	YR1
Hep B GRP TP (B) (N)			0.563		0.104		
Hep B GRP TP (B) (D)			0.855		0.881		
Hep B Indv TP (M) (N)	0.952						0.261
TT Indv TP (B) (N)			0.033	0.361	0.129	0.195	0.643
TT GRP TP (B) (N)			0.497		0.446		
TT GRP TP (B) (D)			0.894		0.779		
TT Indv TP (M) (N)	0.738						0.311
BCG GRP TP (B) (N)		0.230			0.959		
BCG GRP TP (B) (D)		0.958			0.301		
BCG Indv TP (M) (N)	0.153						0.309
BP GRP TP (B) (N)			0.528		0.888		
BP GRP TP (B) (D)			0.888		0.114		
PHA Indv TP (B) (N)	0.273	0.608	0.648	0.490	1.000	0.539	0.523
PHA Indv TP (M) (N)	0.618						0.082
PHA Indv TP (M) (D)	0.007						0.582

5.4 Discussion

The aims of this chapter were to evaluate the effect of MDSC on IFN- γ cytokine producing cells in response to *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), Tetanus toxoid (TT), *Bordetella pertussis* (BP) and Hepatitis B (Hep B) vaccinations and to Phytohaemagglutinin (PHA) in infant-mother pairs. Infants were followed-up from birth, at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age. Mothers on their part were followed-up only at delivery and again at one year postpartum.

IFN- γ cytokine producing cells were quantified in whole PBMC and without MDSC after MDSC depletion using the EasySep[®] magnetic cell separation technique described in [section 4.2.1](#). Total spot forming IFN- γ cytokine producing cells i.e. net spot forming units per million PBMC (Net SFU/10⁶ PBMC) were measured by employing the ELISpot assay described in [section 5.2.1](#). The use of the ELISpot assay to measure cytokine producing cells has been well optimised and is a powerful tool([Slota et al., 2011](#))([Maggioli et al., 2015](#))([Janetzki, 2015](#)). However, we used whole cord blood and peripheral blood mononuclear cells from infants and mothers, which rendered the ELISpot to lack the ability to measure cytokine production at the cellular level. The responses measured are reported as total cytokine responses; we do not know specifically the source of IFN- γ .

The effect of myeloid derived suppressor cells (MDSC) on immune responses can be investigated by evaluating several immune parameters. In [chapter 4](#), we evaluated the effect of MDSC on CD4⁺ T cell proliferation. In this chapter, the effect of MDSC on IFN production was investigated. Cytokines are the molecules that mediate communication between immune cells and vital for evaluating immune function and vaccine immunogenicity([Gasparoni et al., 2003](#))([Peoples et al., 2009](#)). There is limited understanding of the effect of MDSC on cytokine production in the very first few weeks of life in infants, and in mothers postpartum([Gervassi et al., 2014](#)).

Generally, in infants MDSC frequency and absolute count between infants who were responders and non-responders to vaccine induced IFN- γ cytokine producing cells were significantly lower in responders than in non-responders at most time points. However, MDSC

frequency and count varied between responders and non-responders to different vaccine antigens and at different grouped time points.

We hypothesized that MDSC frequency will influence IFN- γ cytokine producing cells to vaccine antigens negatively, in conformity with our hypothesis in infants, this was evident, as MDSC frequency and counts in responders was significantly lower and there were trends towards low quantities of MDSC at most time points in the different vaccine antigen stimulation. A significantly lower MDSC frequency was observed in infants who responded to Hep B induced IFN- γ cytokine producing cells at 10 weeks of age, also evident at 10 and 14 weeks grouped time points, and trends towards low MDSC frequency at 9 months of age in Hep B vaccinated infants and at 10 and 14 weeks grouped time points in TT vaccinated infants. Also, we observed that the MDSC frequency and count in responders to TT increased with age but remained the same in the non-responders. Further, Significantly lower MDSC counts were observed at 6 months post BCG vaccination in infants who responded to BCG stimulation (however the number of non-responders was small), and at 10 and 14 weeks grouped time points in infants who responded to TT stimulation. There was also a trend towards low MDSC counts in infants who responded to Hep B stimulation at 10 weeks of age.

However, there were a few differences between responders and non-responders contrary to our hypothesis as also observed in [Chapter 4, section 4.3.1](#) on CD4⁺ T cell proliferation. In the grouped time point analysis, at 6 and 9 months and one year grouped time points, MDSC frequency was significantly higher in infants who responded to TT stimulation versus the non-responders, and a trend towards high MDSC frequency at one year of age in infants who responded to BCG vaccine stimulation. Also, at 10 weeks of age post BCG vaccination MDSC absolute count was significantly higher in responder than non-responders, but there were few non-responders.

The effect of MDSC on cytokine production to non-specific antigens has been studied previously but there is limited information to vaccines. Rieber *et al.* showed suppression of infant and adult T helper type 1 (Th1), Th2 and Th17 cytokine secretion by cord blood MDSC (Rieber *et al.*, 2013). After stimulations with anti-CD3CD28 (Dynabeads), we have also shown that MDSC

decrease the frequency of IFN- γ producing cells in cord blood of caesarean section delivered neonates(Gervassi *et al.*, 2014). In infants, detectable IFN- γ cytokine producing cells to BCG lasted to approximately 9 months. Cells producing IFN- γ were significantly lower after depleting MDSC at one year of age and a trend towards lower IFN- γ cytokine producing cells to BCG at 6 months of age. The decrease in IFN- γ cytokine producing cells to BCG after MDSC depletion was also evident in the grouped time point analyses at 6, 10 and 14 weeks grouped time points post BCG vaccination in infants as well as at 6 and 9 months and one year, the last follow-up grouped time points. This finding supported our observation of a trend towards high MDSC frequency at one year of age in infants who responded to BCG vaccine stimulation. We think that the difference in the responses observed could be due to the function of the assay. It has been shown that MDSC themselves can make IFN- γ (Matsumura *et al.*, 2012)(Guo *et al.*, 2012)(Cripps *et al.*, 2010) and may depend on the type of antigen stimulation, and may be the reason for the lower IFN- γ producing cells post BCG stimulation.

Detectable IFN- γ cytokine producing cells to TT were observed to approximately one year of age in infants, but there seems to be little effect of MDSC on these responses. IFN- γ cytokine responses to TT were really small and have been fairly undescribed. IFN- γ cytokine may not be an important component for TT vaccination which may be the reason why MDSC had little effect on cells producing IFN- γ . In Italian infants Graziani *et al.* showed that cell-mediated immune responses to TT may be important for long-term immunity after a required vaccine recall(Graziani *et al.*, 2013). Humoral immunity to tetanus vaccination have extensively been described(Siegrist, 2008)(Abramczuk *et al.*, 2011).

Detectable IFN- γ cytokine producing cells to BP vaccination were observed to about 9 months of age, but were not significantly different in whole PBMC and after MDSC depletion in infants. It must be noted that IFN- γ cytokine producing cells to BP in most infants was poorly induced at most time points and most infants did not respond to BP stimulation. In infants, there is evidence of good antibody induction by BP vaccination, and cellular immune responses to BP increased over the course of the vaccination schedule and after booster vaccination(Fadugba *et al.*, 2014)(Zepp *et al.*, 1996)(Edwards *et al.*, 2014).

IFN- γ cytokine producing cells to Hepatitis B vaccination did not significantly differ in whole PBMC and without MDSC in infants in the individual time point analyses. However, in line with our hypothesis after grouping infants at 6 and 9 months and one year grouped time points, IFN- γ cytokine producing cells were significantly higher after MDSC depletion. This observation is in support of our finding of significantly lower MDSC frequency in infants who responded to Hep B induced IFN- γ cytokine producing cells at 10 weeks of age, also evident at 10 and 14 weeks grouped time points, and trends towards low MDSC frequency at 9 months of age in Hep B vaccinated infants who responded post Hep B stimulation. In [Chapter 3](#) we showed that MDSC is significantly present at birth but decreases over time. The quantity of MDSC at 9 months of age may be enough for immune suppression, and after depletion the effect on IFN- γ cytokine producing cells to Hep B was evident. Hep B vaccination have been shown to induce antibody responses([Siegrist, 2008](#))([Spradling et al., 2013](#)) and there is evidence that Hep B induced immunologic T cell in infants are able to trigger specific antibody responses to Hep B later during the vaccination schedule([Bauer et al., 2006](#)).

IFN- γ cytokine producing cells to PHA lasted till one year and the magnitude of responses was high in whole PBMC and after MDSC depletion in infants as expected of a mitogen([Mire-Sluis et al., 1987](#))([Barcellini et al., 1994](#))([Lawn et al., 2007](#)). Contrary to our hypothesis, the effect of MDSC was evident post PHA stimulation, at one year of age IFN- γ cytokine producing cells were significantly lower after MDSC depletion, and this was also observed at 6 and 9 months and one year grouped time points.

In mothers, it is important to note that, it is unlikely that they had been vaccinated against all the vaccines we assessed including BCG, TT, BP and Hep B, as in South Africa pregnant women are not routinely given these vaccines boosters during gestation. The magnitudes of the responses in mothers were often small making it challenging to detect the role of MDSC. After BCG stimulation, there was a trend towards higher IFN- γ cytokine producing cells in the presence of MDSC at one year postpartum, but not after gestation at delivery. We think that with a higher sample size, when the study is completed a significant difference would be evident. Also, we think that the maternal immune system may be in a state of dis-equilibrium between the frequency of Tregs and other regulatory cells including MDSC during gestation, and by one year

postpartum the MDSC level normalised and could be the source of IFN- γ as there is evidence that MDSC can make IFN- γ (Matsumura *et al.*, 2012) (Cripps *et al.*, 2010). Also, the increased IFN- γ production at one year postpartum could be either non-specific or due to TB infection as most of the mothers have not been administered BCG vaccination for about 20 years or more.

Furthermore, in mothers, after gestation at delivery IFN- γ cytokine producing cells to TT stimulation were significantly higher after depleting MDSC. This is in support of our hypothesis, but contrary to our findings in response to BCG where at one year postpartum we observed a trend towards lower IFN- γ cytokine producing cells to BCG stimulation in mothers after removing MDSC. For the first time, we show that after gestation at delivery IFN- γ cytokine producing cells were significantly higher after depleting MDSC in response to TT at delivery. This also shows that maternal MDSC levels during gestation may be influenced by other regulatory cells. MDSC could be suppressed by Tregs during gestation eliminating its effect on IFN- γ cytokine producing cells to BCG after gestation at delivery in mothers. This further, shows that the effect of MDSC on IFN- γ cytokine producing cells may be antigen dependent. Besides the articles by Fujimura *et al.*, there is no direct evidence yet on how Tregs shape the functional phenotype of MDSC to the best of our knowledge. Fujimura *et al.* recently reported that Tregs are maintained by B7-H1 signalling and intern B7-H1 is immunosuppressive on MDSC. This functional crosstalk between Treg and MDSC may play an important role in the induction of immunosuppression by MDSC (Fujimura *et al.*, 2012).

On the contrary, MDSC had little effect on IFN- γ cytokine producing cells to BP and Hep B stimulations in mothers. In South Africa Hepatitis B vaccination was only started 15 years ago, so none of the mothers should have been immunized, due to that any significant differences in Hep B responses would be either non-specific or due to infection. Recently, Pallett *et al.* showed that Hepatitis B virus (HBV) immunopathology was metabolically regulated by MDSC (Pallett *et al.*, 2015) (Weber, 2015). Higher IFN- γ cytokine producing T cells have been shown after depleting MDSC in hepatitis B patients (Pallett *et al.*, 2015) (Weber, 2015). Also, in Hepatitis C virus (HCV) infection, MDSC have been shown to suppress IFN- γ production by CD4⁺ and CD8⁺ T cells (Tacke *et al.*, 2013). The prevalence of Hep B infection in South Africa is highly variable, a study carried out on South African and Botswanan women by Matthews *et al.* and a

systematic review estimating worldwide prevalence of chronic Hep B virus infection in South African women found that about 7% of women had detectable HBsAg(Matthews *et al.*, 2015)(Schweitzer *et al.*, 2015). However, mothers in this study were not tested for Hep B infection.

In mothers, IFN- γ cytokine producing cells to PHA were significantly lower after removing MDSC at delivery, but not at one year postpartum. This is contrary to our hypothesis, but in support of the observation that the source of IFN- γ may be from MDSC, as MDSC have been shown to produce IFN- γ themselves(Matsumura *et al.*, 2012)(Cripps *et al.*, 2010).

As part of our objectives, we evaluated the impact of MDSC in HIV-exposure in infants and HIV infection in mothers on cytokine producing cells. There is no literature to date assessing the suppressive function of MDSC on cytokine production in HIV-exposed uninfected (HEU) infants in their very first few weeks of life to one year of age. We do not know what effect MDSC would have on cytokine responses to vaccine stimulations in HIV+ mothers after gestation at delivery and sometime postpartum.

We have shown that HEU infants have an altered cytokine production profile in response to vaccine and nonspecific antigens(Kidzeru *et al.*, 2014). We showed that *in utero* HIV-exposure, significantly dampens measurable cytokine polyfunctional responses(Kidzeru *et al.*, 2014). The effect of MDSC has also been studied in some clinical conditions in infants. In Chinese children, Pan *et al.* showed that high MDSC frequency in children that presented with recurrent wheezing contributed to high IL-17 cytokine producing cells(Pan *et al.*, 2015). In this study, IFN- γ cytokine producing cells to BCG were generally lower in HEU infants. In support of our hypothesis, others have shown that HIV-exposure contributed to decreased IFN- γ cytokine production, as we show here that IFN- γ cytokine producing cells to BCG were lower in HEU, but was not significant(Hesseling *et al.*, 2015)(van Rie *et al.*, 2006)(Jones *et al.*, 2015).

IFN- γ cytokine producing cells to TT in HEU infants were significantly lower at 10 weeks after the first vaccine dose. However, the number of HEU infants was small. This is in support of our hypothesis that MDSC may be a player in the altered immune system of HEU infants, reducing IFN- γ cytokine producing cells to BCG and TT vaccination(Kidzeru *et al.*, 2014).

Generally, HIV infection had little effect on IFN- γ cytokine producing cells in response to most vaccines stimulations assessed and to PHA. However, after depleting MDSC IFN- γ cytokine producing cells to PHA were significantly higher in HIV+ mothers at delivery. This could be as a result of hyper-activation or reactivation due to HIV infection. In contrast, HIV infection should be immunosuppressive(Hammond *et al.*, 2008). Hammond *et al.* have shown that IFN- γ cytokine producing cells to PPD are suppressed in HIV infected patients(Hammond *et al.*, 2008). Also, contrary to our findings in HIV infection in mothers, Garg *et al.* showed that HIV gp120-expanded MDSC inhibited IFN- γ responses from autologous T cells in HIV+ participants(Garg *et al.*, 2013).

HIV has been well documented for its incredible immune activation much more than other disease states(Paiardini *et al.*, 2013)(Chang *et al.*, 2010)(Catalfamo *et al.*, 2011)(d'Ettorre *et al.*, 2011). The main source of IFN- γ secretion have been shown to be CD4⁺ T cells that are decreased in chronic HIV infected patients due to immune activation(Hammond *et al.*, 2008). Interestingly, we found that the frequency of MDSC at delivery was not significantly different between HIV+ and HIV- mothers but not at one year postpartum (section 3.3.1.2 b). HIV infection may create a hyper-reactive immune environment leading to increased IFN- γ production to non-HIV antigens as observed in HIV infection in the mothers in this study after PHA stimulation. We recommend that further investigations would be warranted.

This study is not without limitations. One limitation is that the number of infants and mothers was small and often volume of blood insufficient for the IFN- γ ELISPOT assay at certain time points. The study is still accruing data and some participants were yet to reach the later follow-up time points. The source of IFN- γ could not be determined as whole PBMC was used. However, this is as a function of the assay, and in future studies Flow cytometry could be used to measure cytokine production at the cellular level using ICS. Also, after depletion using EasySep[®] magnetic cell separation technique, cells seem to be less “happy” compared to cells that have not gone through the same process. This could be one of the reasons why responses decreased after depletion, although the beads are nanoparticles that are not supposed to alter cells functionally in any way(Tomlinson *et al.*, 2013). However, experiments where CD15 enriched cells are added

back to CD15 depleted cells are in progress and should provide a clearer understanding of the effect of the depletion process on IFN- γ cytokine production.

In summary, the findings presented in this chapter suggest that the magnitude of IFN- γ cytokine producing cells to vaccines were generally present in infants to approximately 9 months of age, and the effect of MDSC on IFN- γ cytokine producing cells may be antigen dependent. This antigen dependency may be associated with responses after HIV-exposure in infants. Also, in HIV infection in mothers, IFN- γ cytokine producing cells may be antigen dependent and may be associated with different maternal immune states both at delivery and one year postpartum. Future immune functional studies would be warranted; we think that MDSC may be playing a different role to vaccines and antigens yet to be understood. Also, we think that, the complex immune environment during gestation rich in different immune regulatory cells in the mothers may suppress MDSC's effect.

5.5 References

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CHAPTER 6

CONCLUDING REMARKS

In this thesis, our objectives were to evaluate the quantity and ontogeny of MDSC in [Chapter 3](#), the effect of MDSC on T cell proliferation in [Chapter 4](#), and in [Chapter 5](#) MDSC's effect on cytokine production in infants and mothers from birth to one year postpartum.

In [Chapter 3](#), we found a significant positive correlation between infants and maternal MDSC frequencies and absolute counts both at delivery and one year postpartum. This illustrates that the quantity of infants' MDSC is directly associated to maternal MDSC immediately after gestation at delivery and through time postpartum as seen at one year. This implies that factors responsible for MDSC induction in infants and mothers is similar, and suggests that this may be an in utero event, such as the degree of materno-fetal mismatch. This is supported by evidence in mice, whereby MDSC are crucial for maintaining fetomaternal tolerance([Pan et al., 2016](#)).

As expected, we found that the quantity of MDSC in cord and peripheral blood was not significantly different in infants at birth, even though the cord blood samples show a definite trend for reduced MDSC frequency. This finding demonstrates that the immune composition of cord and peripheral blood in infants at birth is similar. However, since to the best of our knowledge this is the first study to perform such analysis, we recommend further experiments and with a higher sample size to confirm the finding. In line with our hypothesis, MDSC frequency and counts were highest at birth, and declined over time. From birth through 6, 10 and 14 weeks, and to 6 and 9 months, there was a significant decrease in MDSC frequency in infants. We think that, the tolerance and anti-inflammatory environment during gestation necessary to provide for full-term viviparity, may persist postnatally and wane rapidly postpartum([Nair et al., 2015](#))([Makrigiannakis et al., 2008](#))([Makrigiannakis et al., 2011](#))([Bansal, 2010](#)).

We also found that in mothers, MDSC frequency was significantly higher at delivery compared to one year postpartum. In line with our expectation, this finding further reiterates that these cells are present in both maternal and fetal circulation to maintain tolerance. Our findings illustrate

that pregnancy as a condition favours the accumulation of MDSC in mothers, and further persist in infants after birth, and may be immunosuppressive. In support of our finding, we have previously shown that granulocytic MDSC in infants were similar to those reported in peripheral blood of cancer patients and are immunosuppressive (Gervassi *et al.*, 2014). These cells have been well described in other conditions including in cancer patients and very few can be detected in healthy adults but the frequency increase with ageing (Gabrilovich *et al.*, 2012) (Gantt *et al.*, 2014) (Verschoor *et al.*, 2013) (Brandau *et al.*, 2011) (Khaled *et al.*, 2014) (Gabitass *et al.*, 2011).

HIV-exposure had little effect on MDSC frequency, and as discussed in Chapter 3, this may be because HEU infants though are exposed to HIV infection *in-utero* but are not infected with or directly exposed to viral antigens such as gp120. There is substantial evidence that HIV infection is associated with high MDSC levels in infected individuals (Vollbrecht *et al.*, 2012) (Garg *et al.*, 2013). Although studies have evaluated the effect of HIV infection on MDSC in adults (Vollbrecht *et al.*, 2012) (Garg *et al.*, 2013), to the best of our knowledge they have not investigated the effect in mothers postpartum. We found no significant difference in MDSC frequency between HIV+ versus HIV- mothers at delivery. However, in accordance with our hypothesis, at one year postpartum, HIV+ mothers presented with a significantly higher MDSC frequency compared to their uninfected counterparts. This is not surprising since high levels of MDSC are described in disease states (Tacke *et al.*, 2013) (Qin *et al.*, 2013) (Gama *et al.*, 2012) (Vollbrecht *et al.*, 2012) (Garg *et al.*, 2013) (du Plessis *et al.*, 2013).

This is to our knowledge, the first time that the effect of MDSC on antigen-specific responses has been evaluated. In Chapter 4, there were no significant differences in MDSC frequency and absolute count between infants whose CD4⁺ T cells responded and those who had poor CD4⁺ T cell proliferation to antigens. However, there was a trend towards high MDSC counts in responders compared to the non-responders to Hep B vaccine stimulation.

Likewise, MDSC had little effect on T cell proliferation in response to vaccine antigens including Hep B, TT and PPD at most time points in whole PBMC versus after MDSC depletion in infants. However, in response to non-specific stimulation with anti-CD3CD28 (Dynabead) in infants CD4⁺ T cell proliferation in whole PBMC was significantly higher compared to after

MDSC depletion at birth and at 14 weeks of age. In contrast to this finding, our group and others have previously observed high MDSC frequency in cord blood that suppressed T cell responses to non-specific antigens(Rieber *et al.*, 2013)(Gervassi *et al.*, 2014). In mothers, though they had not been vaccinated with the vaccine antigens assessed for a long time, we found significantly higher CD4⁺ T cell proliferative responses to Hep B and TT vaccine stimulation after MDSC depletion compared to whole PBMC at delivery but not at one year postpartum. At one year postpartum, maternal Dynabead-induced CD4⁺ T cell proliferation was significantly higher after MDSC depletion versus in whole PBMC. This is opposite to the effects we saw in infants on Dynabead-induced T cell proliferation, and suggest that MDSC may be functionally distinct in neonates versus in mothers. As discussed in Chapter 4, several factors could be responsible for the lack of significant differences in CD4⁺ T cell proliferation to vaccines in whole PBMC and after MDSC depletion including the fact that MDSC depletion was not always 100% and there were not always enough cells to do the assays. In Chapter 3, we found that MDSC are elevated at birth and rapidly decrease from 6 weeks onwards, this may account for the lack of effect of MDSC on T cell proliferation to specific antigens in infants. Since we could not test responses to antigens other than Dynabeads in infants at birth (when they are antigen-naïve), the effect of MDSC could not be assessed. On the other hand, in mothers, who are antigen experienced, we could assess responses at delivery when MDSC frequency was highest and the effects were likely more apparent.

HIV-exposure in infants and maternal HIV infection had little effect on T cell proliferation to vaccine antigens at most time points neither in whole PBMC in the presence of MDSC nor after MDSC removal. The lack of significant differences was also observed after stimulation with Dynabead in both infants and mothers. The findings are not surprising in the HIV-exposed infants, since we saw no differences in MDSC number and frequency in these infants. In mothers with HIV-infection, the lack of effect of MDSC may be because MDSC are not functionally affected by HIV infection, but rather instead just increased in frequency. Another possible reason may be due to the limited number of samples for which responses were measured. However, there is evidence that MDSC suppress T cell proliferation in HIV-infected individuals(Qin *et al.*, 2013)(Gama *et al.*, 2012)(Nagaraj *et al.*, 2013)(Vollbrecht *et al.*, 2012).

In [Chapter 5](#), we evaluated the effect of MDSC on IFN- γ cytokine producing cells to vaccines. Generally, in infants MDSC frequency and absolute count between infants who were responders and non-responders to vaccine induced IFN- γ cytokine producing cells were significantly lower in responders than in non-responders at most time points, as hypothesized, implying that MDSC do indeed have suppressive effects on Th1 cytokine production at least to protein antigens. This finding was confirmed by our experiments evaluating the impact of MDSC on IFN- γ cytokine producing cells by MDSC depletion to most vaccine antigens. IFN- γ cytokine producing cells to Hepatitis B at the later grouped time points (6, 9 and 12 months) were significantly higher after MDSC depletion. This observation is in support of our finding of significantly lower MDSC frequency in infants who responded to Hep B in IFN- γ cytokine production. Hep B vaccination have been shown to induce antibody responses([Siegrist, 2008](#))([Spradling et al., 2013](#)) and there is evidence that Hep B induced immunologic T cell in infants are able to trigger specific antibody responses to Hep B later during the vaccination schedule([Bauer et al., 2006](#)). Although frequency of MDSC was lower in non-responders to TT at later time points, absolute counts of MDSC in infants who responded to TT a similar antigen to Hep B in that it is a protein, albeit with a different adjuvant was significantly higher in non-responders.

On the other hand, detectable IFN- γ production to BCG was significantly lower after depleting MDSC at one year of age, also evident in the grouped time point analyses and a trend towards lower IFN- γ cytokine producing cells to BCG at 9 months of age. Detectable IFN- γ cytokine producing cells to BCG lasted until approximately 9 months and were more robust than those to Hep B, TT and BP. It is possible that the difference in the responses observed could be due to the function of the assay. It has been shown that MDSC themselves can make IFN- γ ([Matsumura et al., 2012](#))([Guo et al., 2012](#))([Cripps et al., 2010](#)) and this may depend on the type of antigen stimulation present, and may be the reason for the lower IFN- γ producing cells post BCG stimulation. On the other hand, the effect of MDSC on BCG may indeed be different to that of Hep B and TT, as BCG is a live-attenuated vaccine that is known to be particularly successfully immunogenic in infants([Ota et al., 2002](#)).

Similarly, at one year of age IFN- γ cytokine producing cells were significantly lower after MDSC depletion after PHA stimulation, also evident in the grouped time point's analyses. The possible reason for this is that MDSC could have a different function in pregnancy than post pregnancy where the maternal immune state may be normalized post gestation at a later time point at one year. This effect may be highly felt in non-specific and stronger stimulation. Most studies have investigated the impact of MDSC on non-specific antigens on Dynabead. Contrary to this finding, our group and others have previously observed suppressed IFN- γ responses to Dynabead by MDSC(Rieber *et al.*, 2013)(Gervassi *et al.*, 2014). The possible reason for this may be that, the mechanism by which PHA and Dynabead stimulate IFN- γ responses may be different, and warrant further investigation.

IFN- γ cytokine producing cells to TT in whole PBMC were significantly lower in HEU infants at 10 weeks after vaccination in whole PBMC. This is in conformity with our finding in a previous study and that of others that HIV-exposure may have a negative effect on cytokine responses(Kidzeru *et al.*, 2014)(Hesseling *et al.*, 2015)(van Rie *et al.*, 2006)(Jones *et al.*, 2015). However, no significant differences were observed between HEU and HU infants in other antigen stimulations. Our data illustrates that in whole PBMC in the presence of MDSC in infants, IFN- γ cytokine production varies between vaccine antigens and is also dependent on the infant age. Also, there was limited number of HEU infants for which IFN- γ cytokine producing cells could be measured. HIV infection also had little effect on IFN- γ cytokine producing cells in whole PBMC in the presence of MDSC in response to all the vaccines assessed. After depleting MDSC IFN- γ cytokine producing cells to PHA were significantly higher in HIV+ mothers at delivery, but not at one year postpartum. These findings suggest that MDSC may be functionally different during HIV infection in terms of their effects on IFN- γ .

In this study, we evaluated the effects of MDSC on IFN- γ responses. It would be important to measure other cytokines. The effect of MDSC has also been studied in some clinical conditions in infants evaluating other cytokines. In Chinese children, Pan *et al.* showed that high MDSC frequency that presented with recurrent wheezing contributed to high IL-17 cytokine producing cells(Pan *et al.*, 2015). We have stored collected plasmas and supernatants from which other cytokines would be measured by ELISA and Luminex analysis in future. Also, other suspected

immunosuppressive mechanisms other than Arginase-1 (ARG) production could be explored in future including the evaluation of the quantity of reactive oxygen species (ROS) as well as nitric oxide synthase (NOS). The quantity of ROS and NOS could be evaluated with their respective antibodies for ELISA in plasmas from the infants(Wu *et al.*, 2011)(Held, 2012)(Sun *et al.*, 2003). We would also recommend future studies to consider measuring the expression of these factors in the cells using flow cytometry(Jia *et al.*, 2010) (Jayaraman *et al.*, 2012).

In summary, the results from this dissertation show that MDSC may have an influence on early childhood vaccines, and that may be vaccine and antigen dependent. These cells may therefore play a partial role in the increased immune vulnerability in infants in early life. Consideration of these effects of these and other immunoregulatory cells should be taken into consideration when designing vaccines for neonates.

6.2 References

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APPENDICES

Appendix 3.1: Correlation between the frequencies and counts of MDSC versus neutrophil, maternal MDSC, infant weight and gestation age

We obtained full blood counts (FBC) from infants at birth and at 6 months of age. The maternal MDSC at birth and one year postnatal was obtained during follow-up, and the infant's weights and gestational ages were obtained as part of the cohort characteristics.

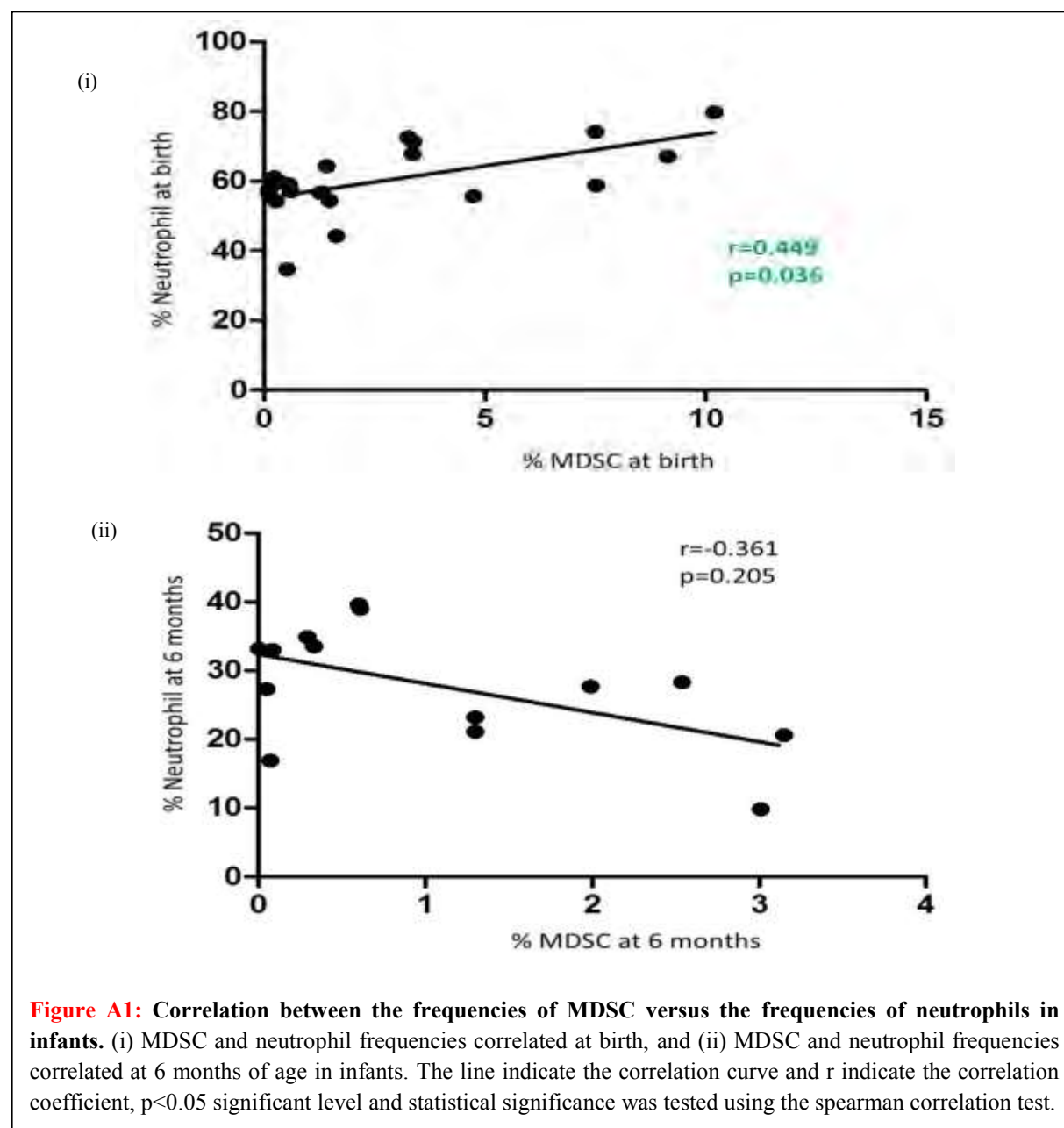
Spearman correlations were performed between MDSC versus neutrophil frequencies and counts at birth and later at 6 months of life [Figures A1(i-ii)] and [Figure B1(i-ii)].

Spearman correlations were performed between infants MDSC and infant's weights at the follow-up time points i.e. at birth, 6, 10 and 14 weeks, at 6 and 9 months and one year of age [Figures A2(i-vii)] and [Figure B2(i-vii)].

Furthermore, we performed Spearman correlations between infants MDSC at all time points versus infant gestational age [Figures A3(i-vii)] and [Figure B3(i-vii)].

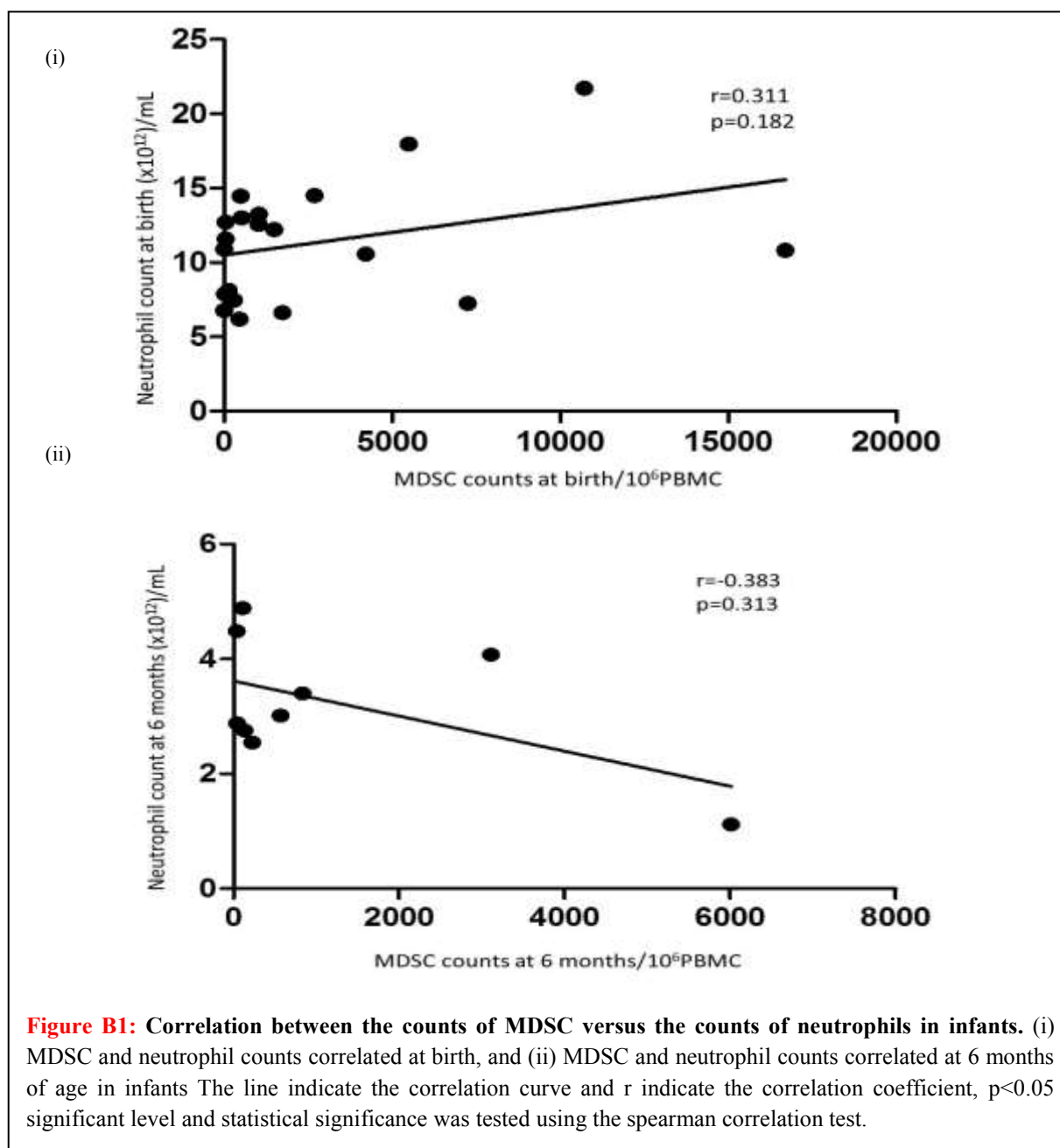
(i) Correlation between the frequencies of MDSC versus the frequencies of neutrophils in infants

At birth, there was a significant positive correlation between the frequencies of MDSC versus neutrophils at birth [Figure A1(i)]. While at 6 months of age, there was a negative correlation but was not significant [Figure A1(ii)].



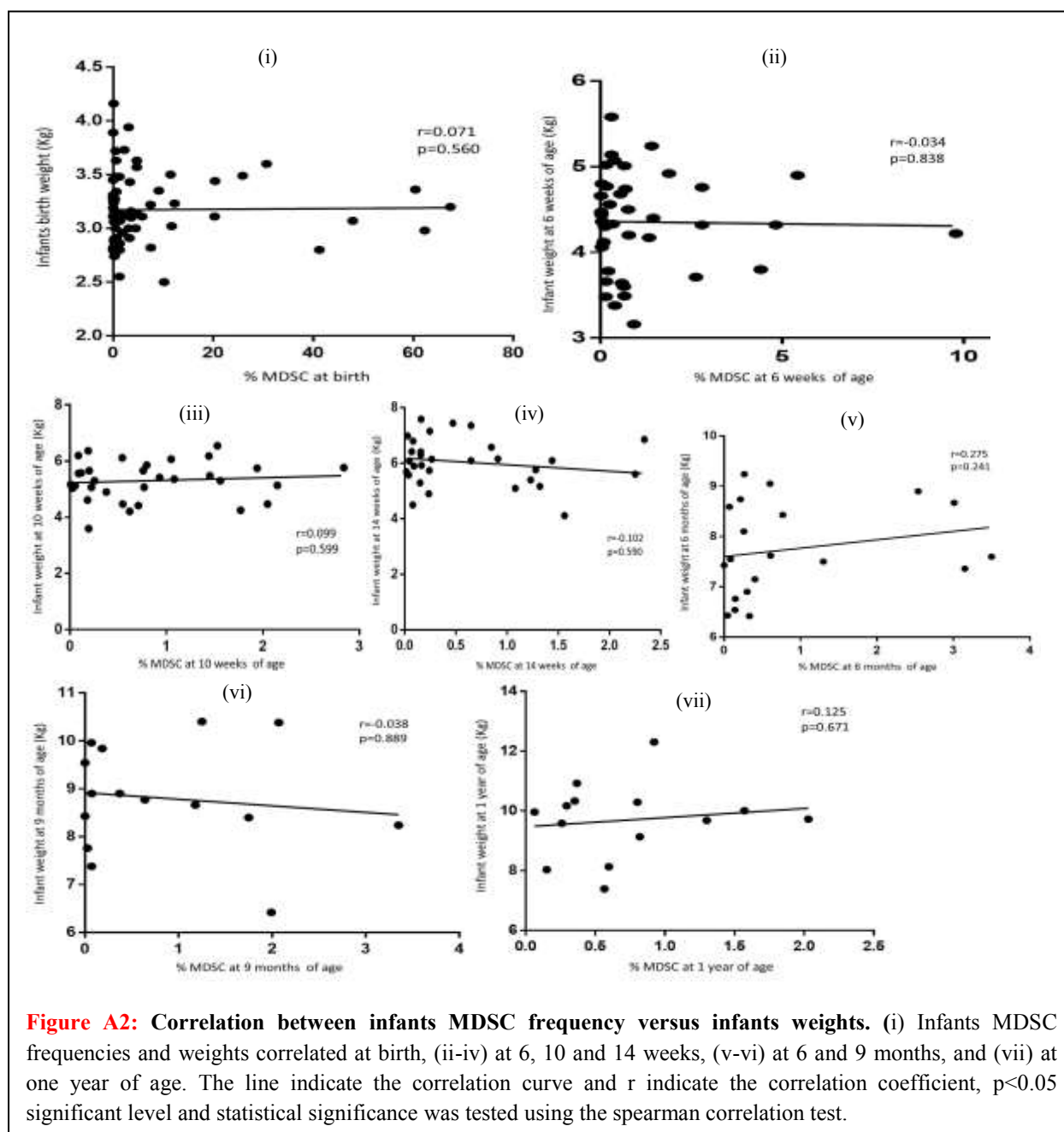
(ii) Correlation between the counts of MDSC versus the counts of neutrophils in infants

At birth, there was a positive correlation between the counts of MDSC versus neutrophils at birth [Figure B1(i)], while at 6 months of age, there was a negative correlation [Figure B1(ii)]. However, these correlations were not significant.



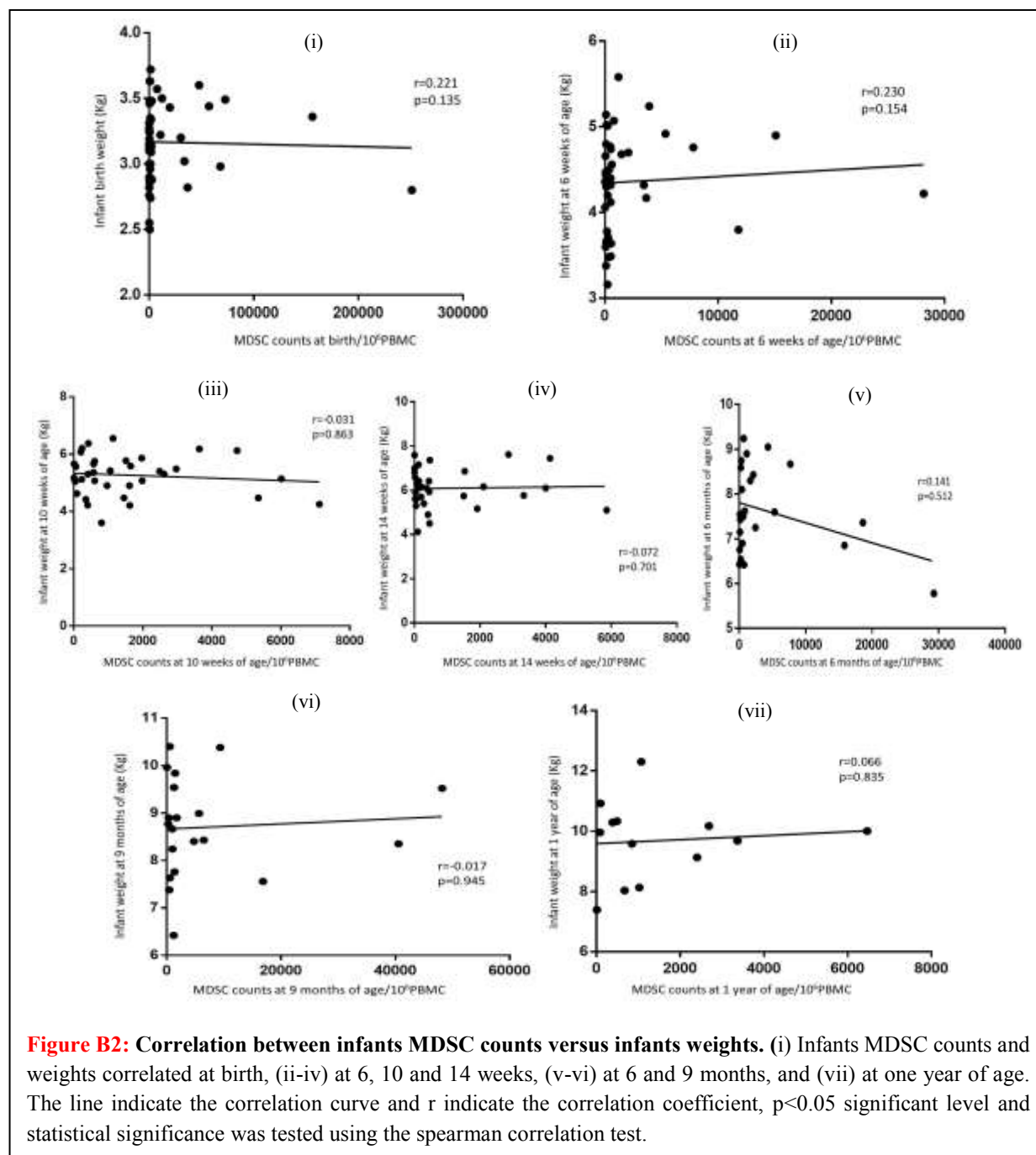
(v) Correlation between the infants MDSC frequencies versus infants weights

At birth, there was no significant correlation between the infants MDSC frequencies versus birth weights [Figure A2(i)]. This lack of significant correlation between MDSC frequency and infants weights was also observed at other time points [Figure A2(ii-vii)].



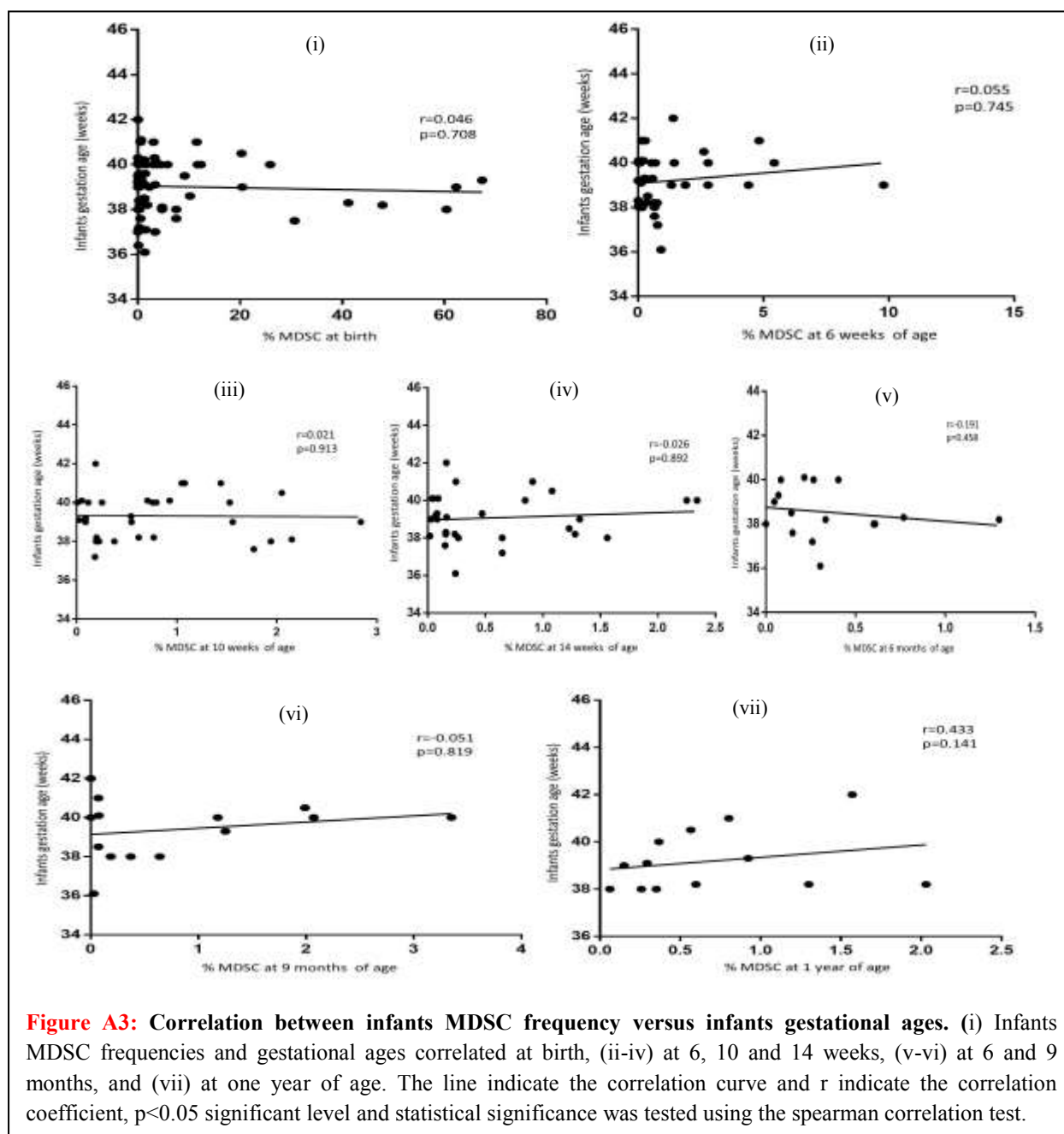
(vi) Correlation between the infants MDSC counts versus infants weights

At birth, there was no significant correlation between the infants MDSC counts versus birth weights [Figure B2(i)]. This lack of significant correlation between MDSC counts and infants weights was also observed at other time points [Figure B2(ii-vii)].



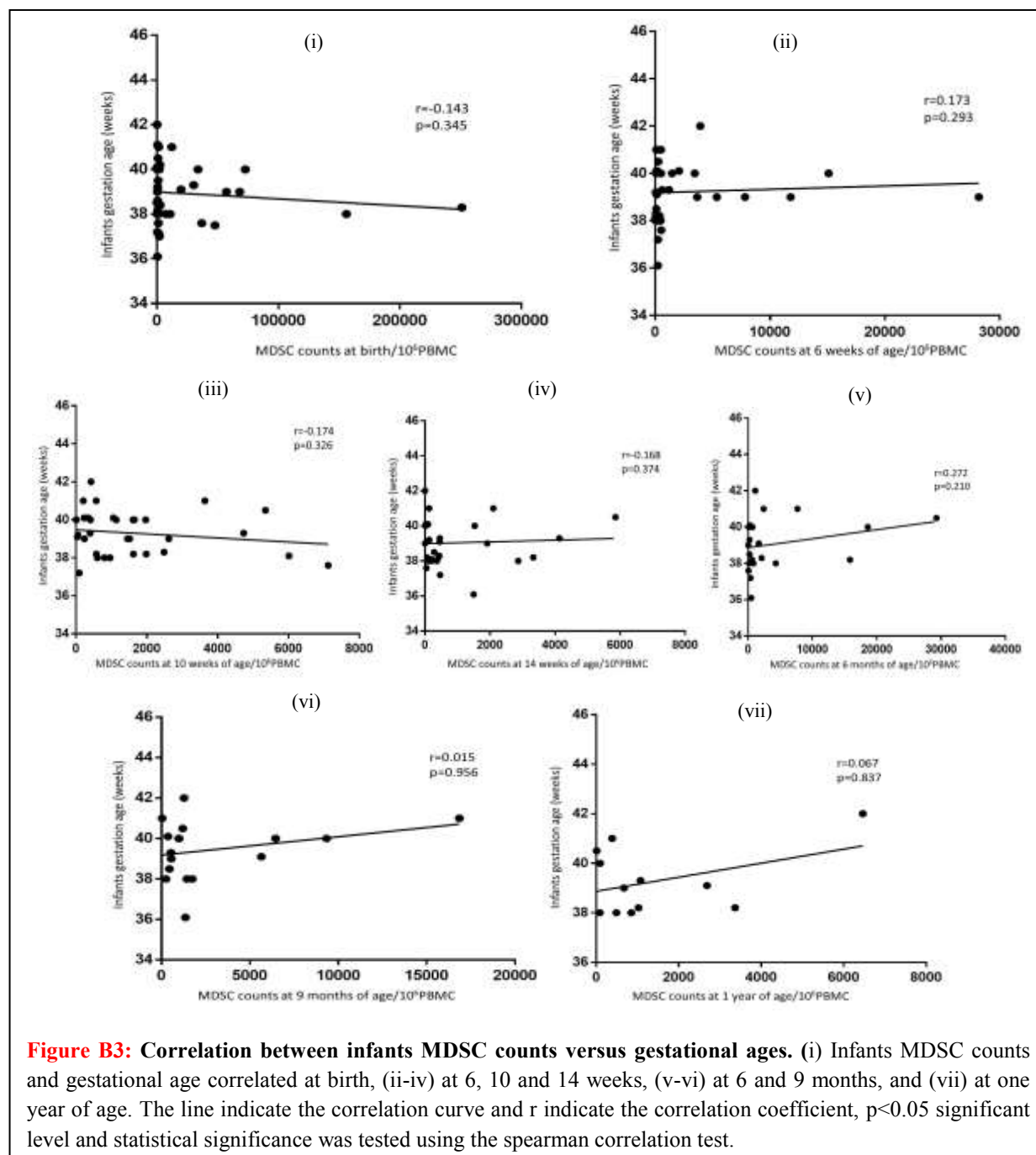
(vii) Correlation between the infants MDSC frequencies versus gestational age

At birth, there was no significant correlation between the infants MDSC frequencies versus gestational ages [Figure A3(i)]. This lack of significant correlation between MDSC frequency and infants gestational ages was also observed at other time points [Figure A3(ii-vii)].



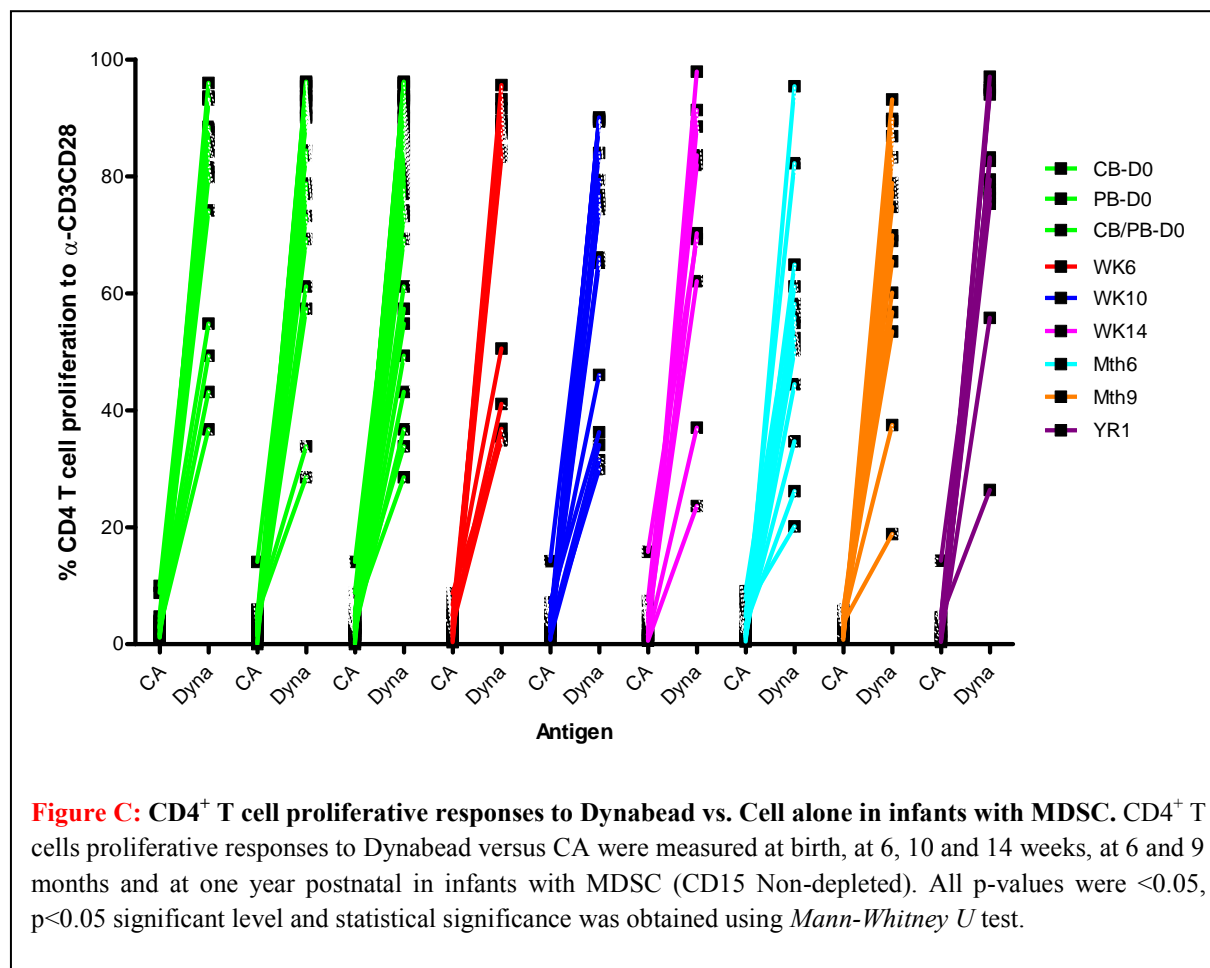
(viii) Correlation between the infants MDSC counts versus gestational ages

At birth, there was no significant correlation between the infants MDSC counts versus gestational ages [Figure B3(i)]. This lack of significant correlation between MDSC counts and infants gestational age was also observed at other time points [Figure B3(ii-vii)].

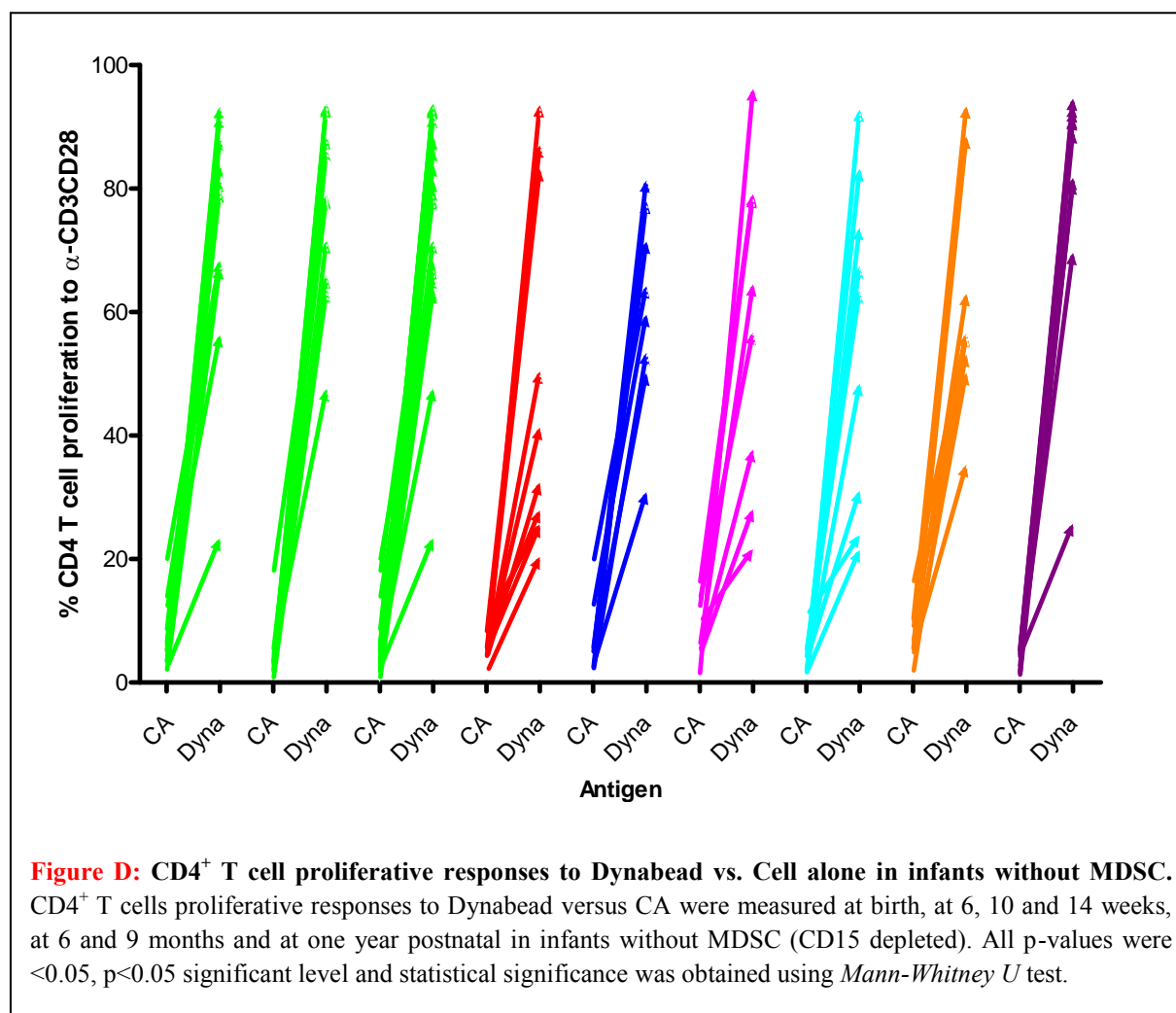


Appendix 4.1: CFSE proliferation assay validity

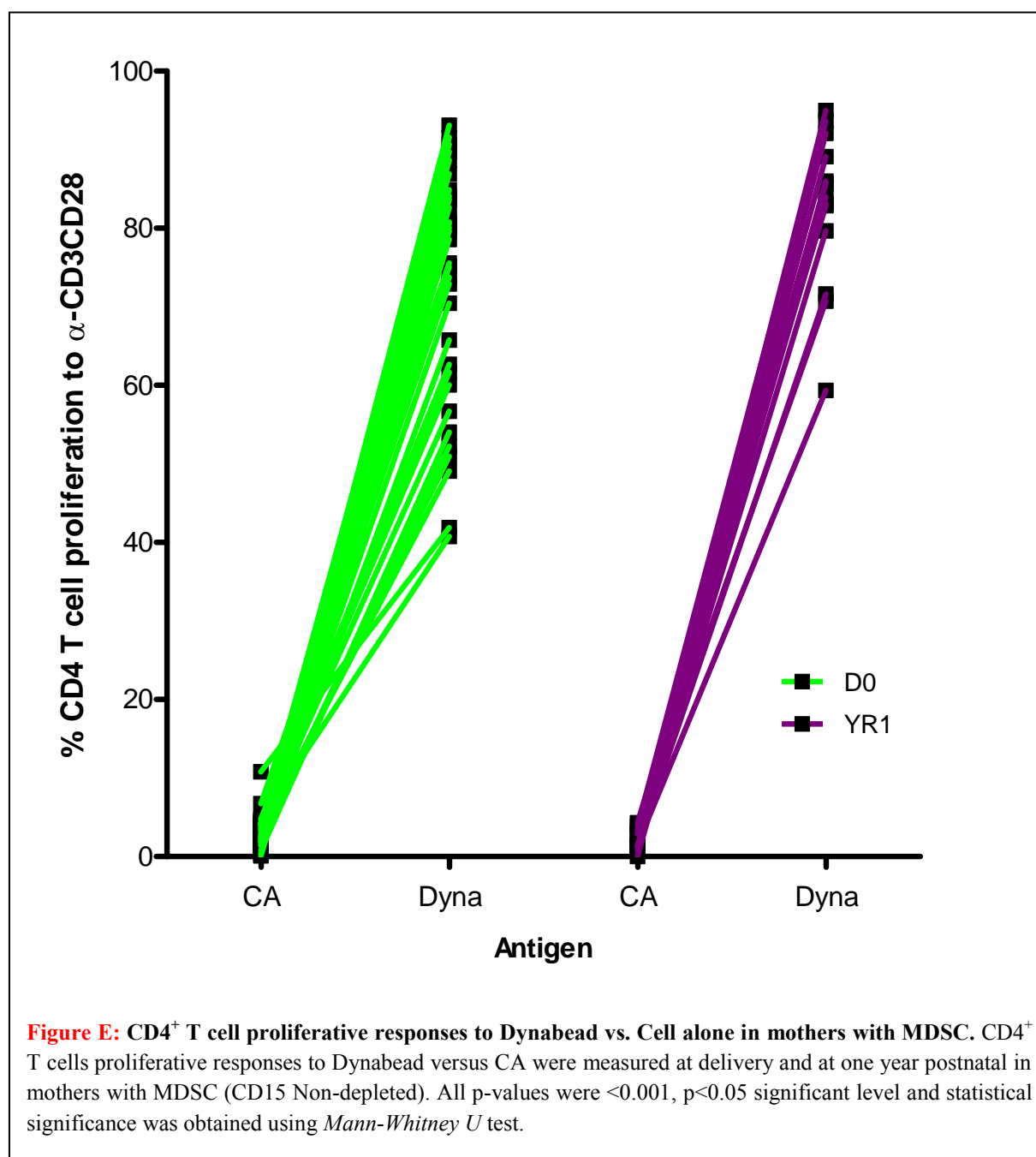
(i) Dynabead (Dyna) (anti-CD3CD28) causes CD4 T-cell proliferation in infants vs. Cells alone (CA) with MDSC



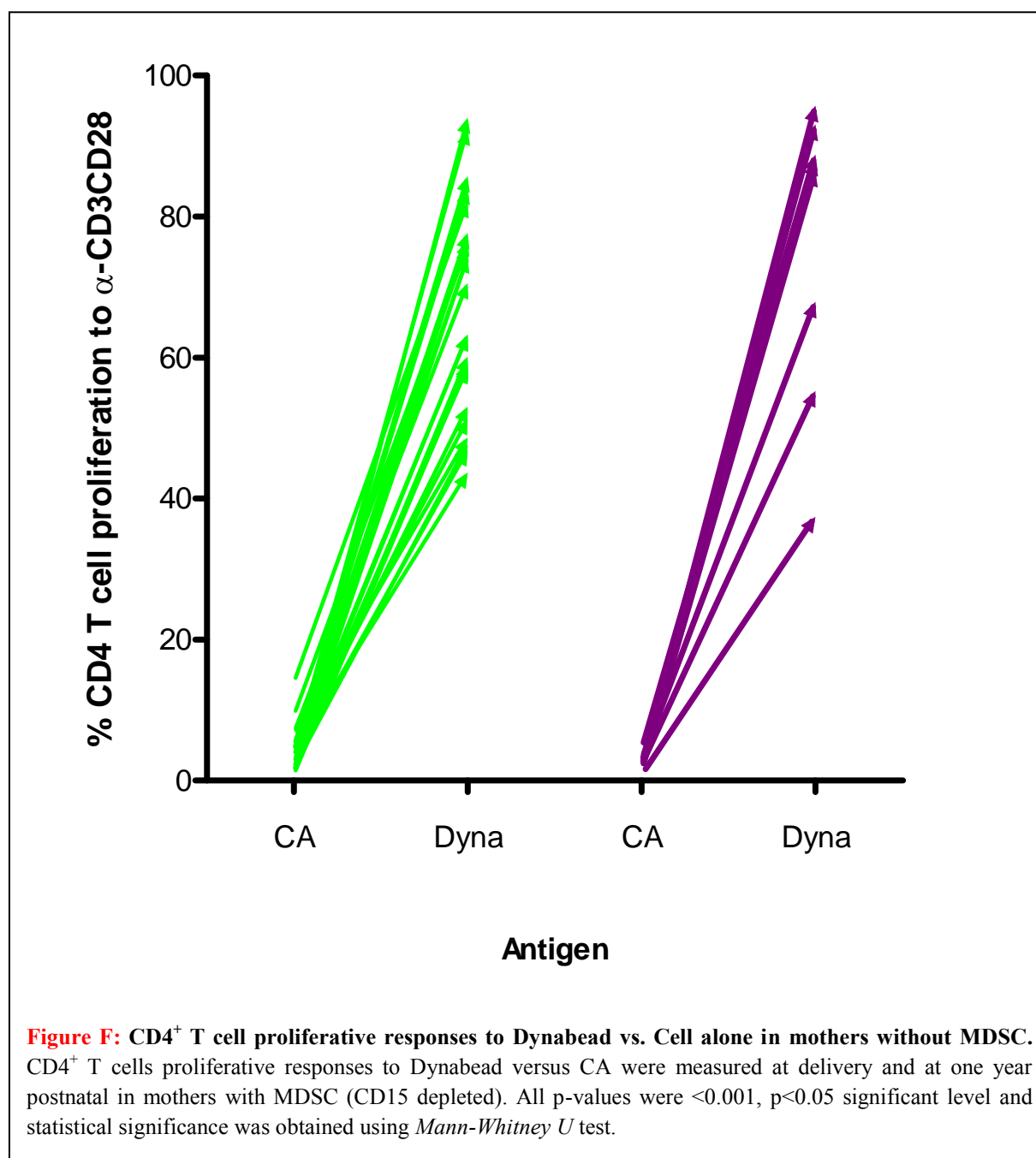
(ii) Dynabead (Dyna) (anti-CD3CD28) causes CD4 T-cell proliferation in infants vs. Cells alone (CA) without MDSC



(iii) Dynabead (Dyna) (anti-CD3CD28) causes CD4 T-cell proliferation in mothers vs. Cells alone (CA) with MDSC



(iv) Dynabead (Dyna) (anti-CD3CD28) causes CD4 T-cell proliferation in mothers vs. Cells alone (CA) without MDSC

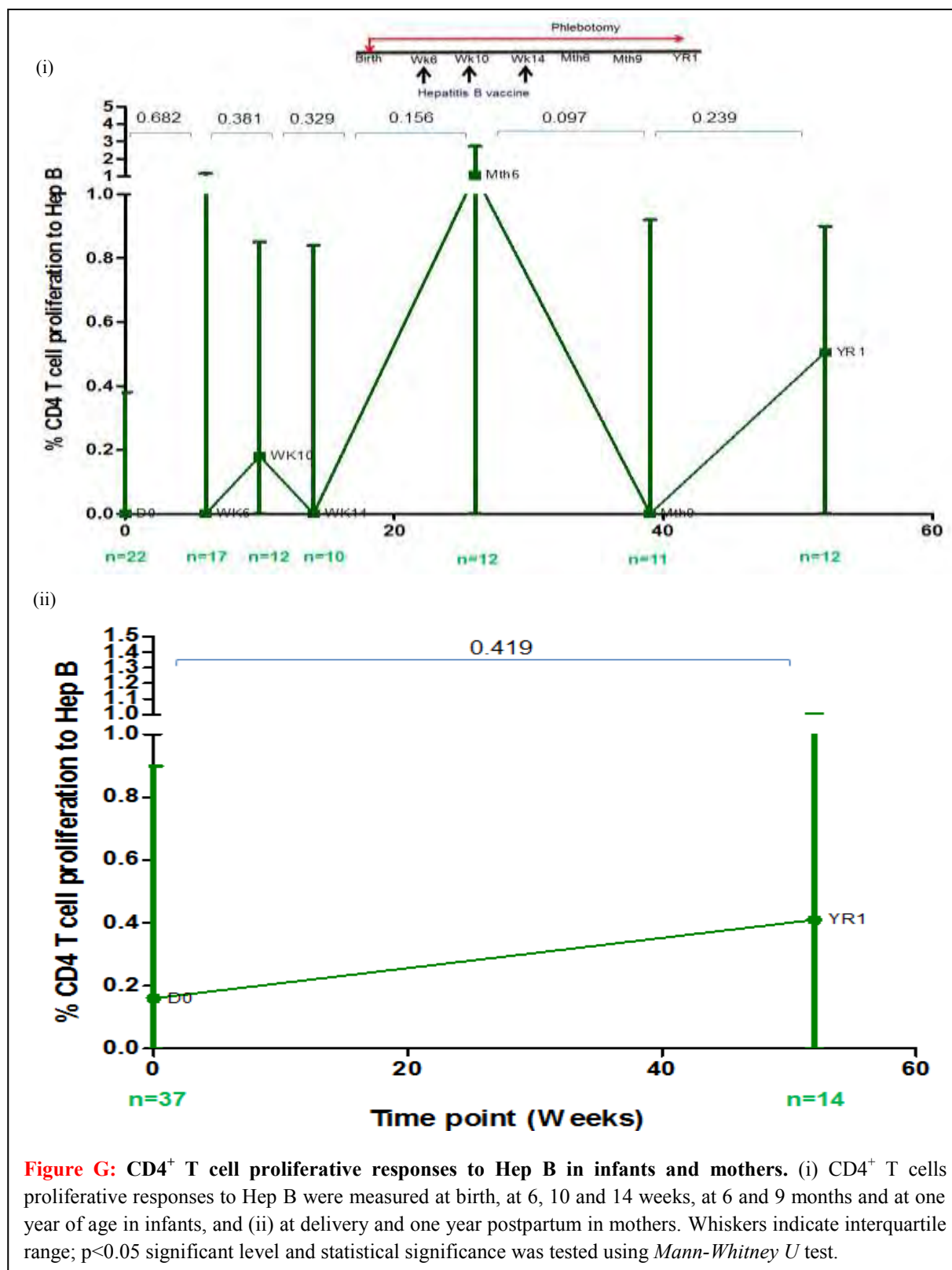


Appendix 4.2: T cell proliferative responses to vaccines in infants and mothers through time

Generally, vaccine responses varied in the different antigens through time. Some responses to vaccines were stronger and some low. Also, responses varied between infants and their mothers. Below is the general dynamic in vaccine responses (median frequency of proliferating cells) to the different antigens in infants and mothers through time regardless of MDSC.

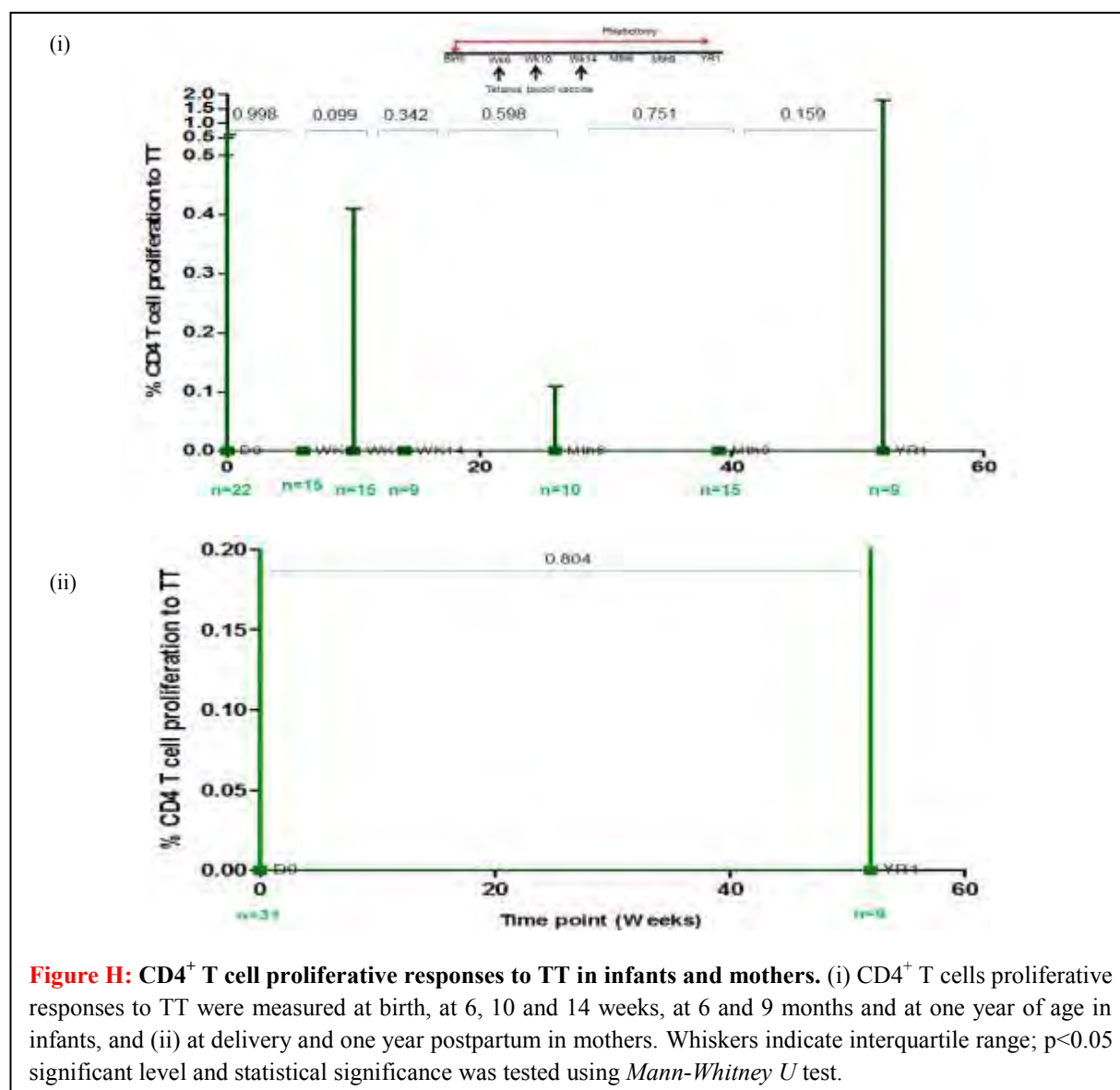
(i) T cell proliferative responses to vaccines in infants and mothers through time after Hep B stimulation

Generally, Hep B responses peaked at 10 weeks after priming at 6 weeks in infants and then dropped at 14 weeks post second vaccine dose. The responses then further rose after the last and third vaccine dose at 14 weeks then varied till one year of age [Figure G(i)]. While in the mothers, Figure G(ii), Hep B responses increased from delivery to one year postpartum.



(ii) T cell proliferative responses to vaccines in infants and mothers through time after TT stimulation

Generally, TT responses were low throughout, from priming at 6 weeks of age, through 10 weeks in infants and also, at 14 weeks post second vaccine dose. The responses then further remained low at 9 months and one year of age [Figure H(i)]. Likewise in the mothers, Figure H(ii), TT responses were low from delivery to one year postpartum.



(iii) T cell proliferative responses to vaccines in infants and mothers through time after PPD stimulation

Generally, PPD responses peaked between 6, 10 and 14 weeks after BCG vaccination at birth in infants and then dropped by 6 months of age. The responses then rose again at 6 months through 9 months to one year of age [Figure I(i)]. While in the mothers, Figure I(ii), PPD responses increased from delivery to one year postpartum.

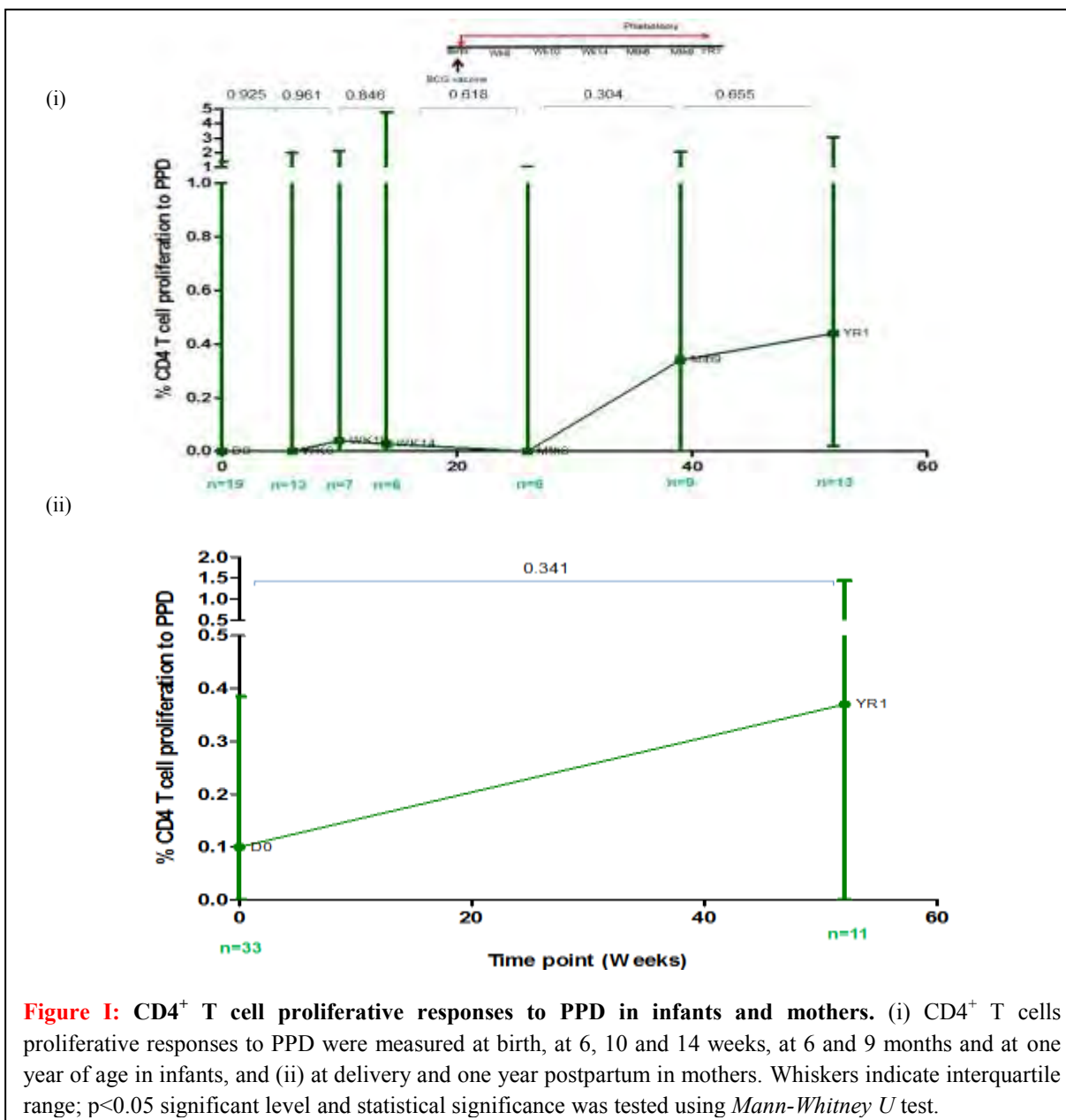


Figure I: CD4⁺ T cell proliferative responses to PPD in infants and mothers. (i) CD4⁺ T cells proliferative responses to PPD were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and (ii) at delivery and one year postpartum in mothers. Whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

(iv) T cell proliferative responses to Dynabead (α -CD3CD28) in infants and mothers through time

Generally, Dynabead responses as expected were strong from birth through 6, 10 and 14 weeks in infants till 6 and 9 months of age. The responses increased with age and were significantly higher by one year of age [Figure J(i)]. While in the mothers, Figure J(ii), Dynabead responses increased from delivery to one year postpartum.

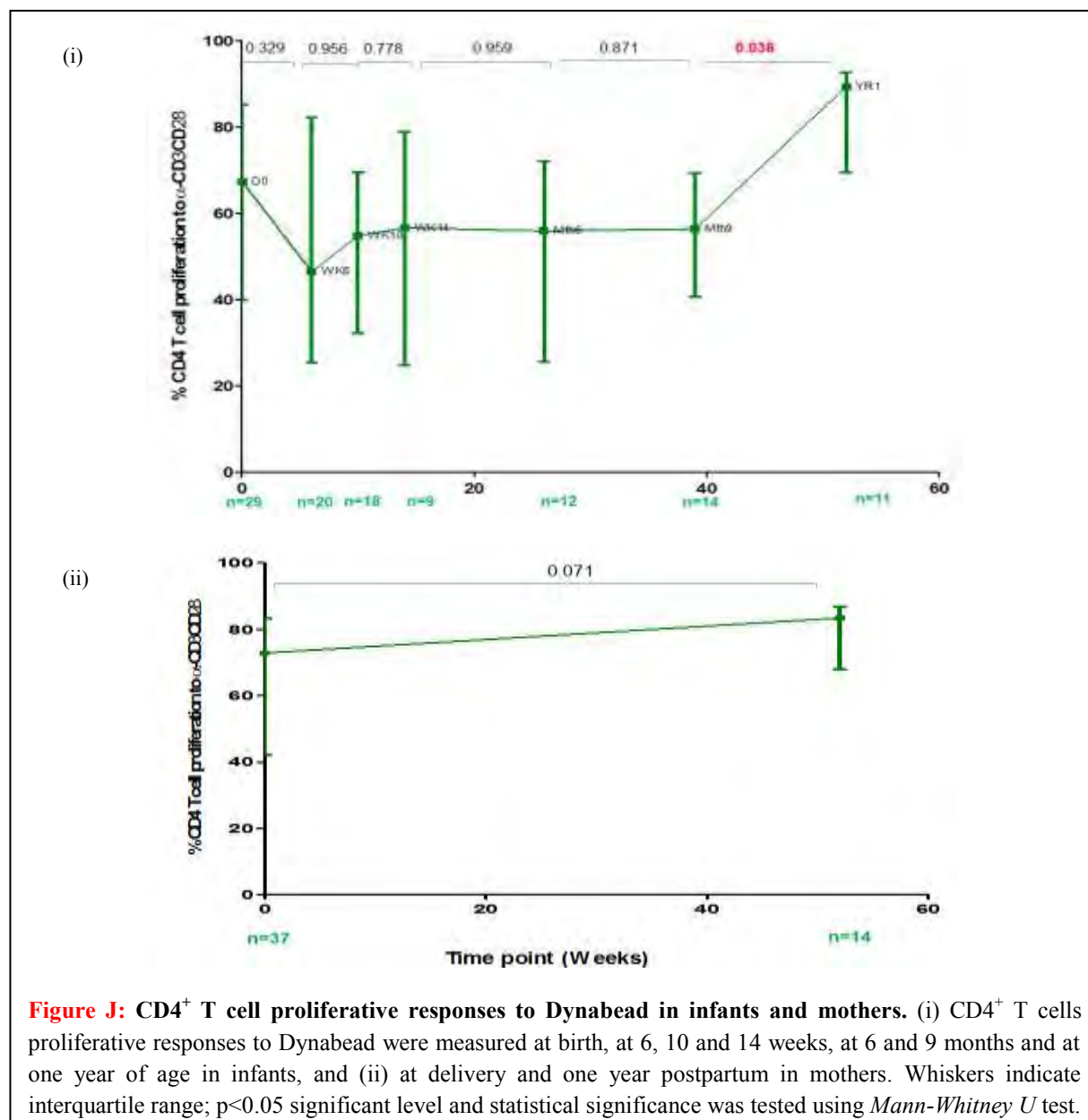
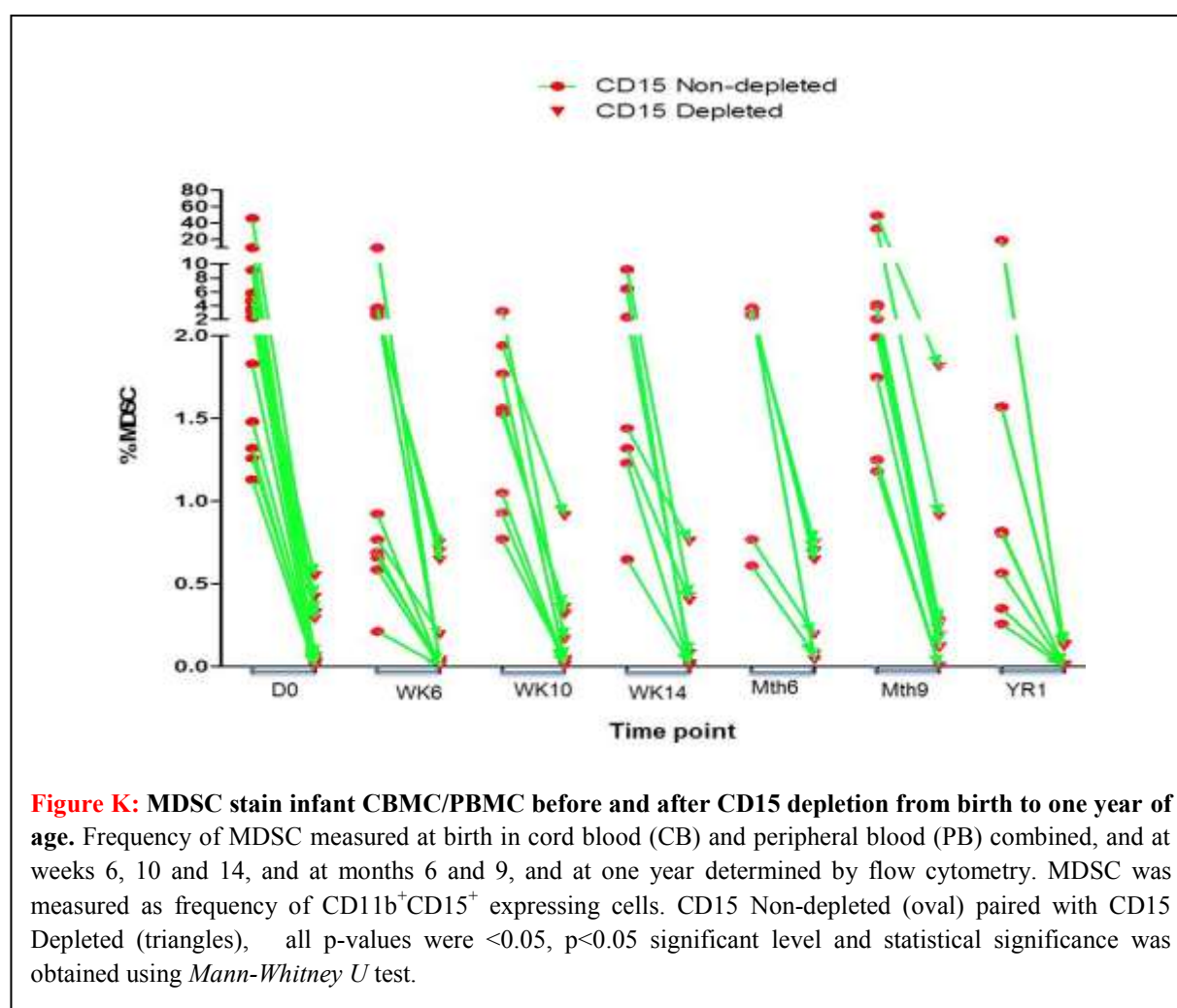


Figure J: CD4⁺ T cell proliferative responses to Dynabead in infants and mothers. (i) CD4⁺ T cells proliferative responses to Dynabead were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and (ii) at delivery and one year postpartum in mothers. Whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

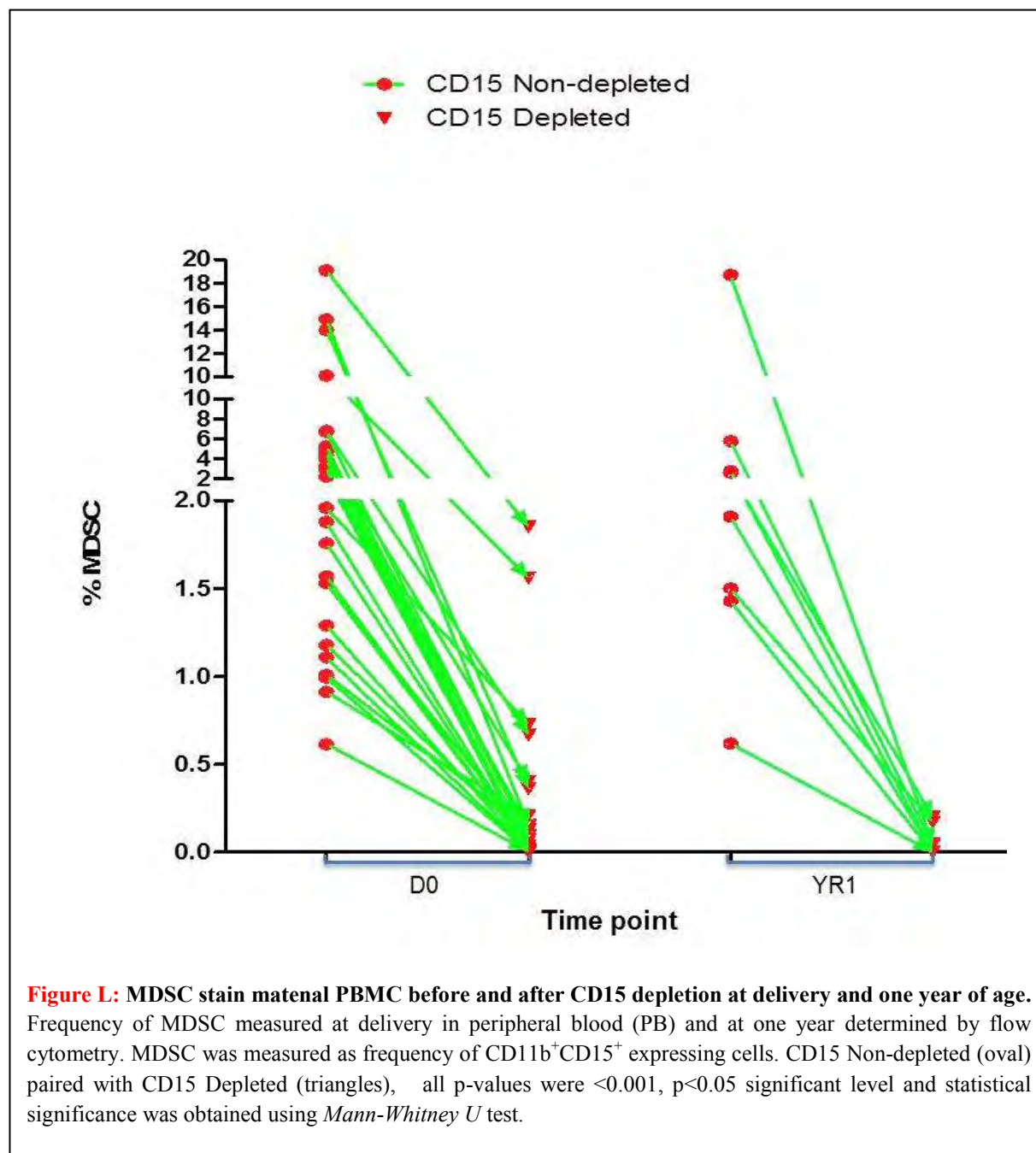
Appendix 4.3: CD15-depletion purity

Since, MDSC highly express CD15, selectively depletion of MDSC was done using EasySep[®] magnetic cell separation technique (Section 4.2.1) and the cells were stained for flow cytometry in a 96 well round bottom plate. After sample acquisition by flow cytometry, the flow files were analyzed using FlowJo v9.4.7 (Tree Star). Comparison of CD4⁺ T cell proliferation and IFN- γ cytokine producing cells to vaccines and antigens in whole PBMC and after MDSC depletion was only done when the CD15-depletion purity was 90% and above for samples to be included in the data for further analysis.

(i) Validation of MDSC depletion in infants



(ii) Validation of MDSC depletion in mothers



Appendix 4.4: CD4⁺ T cell proliferation to vaccines and antigens in unmatched whole PBMC (presence of MDSC) versus without MDSC (after MDSC depletion) in all infants and mothers enrolled from who whole blood samples were collected

The T cell proliferative responses to HepB and TT vaccinations and to PPD and Dynabead antigens in CBMC and/or PBMC were evaluated in infants and mothers in the presence and absence of MDSC, to evaluate the effects of MDSC more directly (Figure 4.1). Since MDSC highly express CD15, as described in section 4.2.1, the EasySep[®] magnetic cell separation technique was performed to deplete CD15⁺ expressing cells from a portion of the CBMC and/or PBMC. The effect of MDSC on T cell proliferative responses to vaccines and antigens was then measured by performing the CFSE incorporation technique on cells depleted of CD15⁺ cells (absence of MDSC) and in whole PBMC (presence of MDSC) (Figure 4.2). Among the MDSC depleted sample group, only samples with depletion purity of 90% or more were included in the analysis (Appendix 4.3).

As described in section 4.3 (2), T cell proliferative responses were measured in infants at all individual time points post vaccination (i.e. at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age), and in mothers, the T cell proliferation was performed at delivery and again at one year postpartum. We also evaluated the ontogeny of the effect of MDSC on CD4⁺ T cell proliferation to vaccines and antigens in a grouped analyses, based on the time points the different vaccines assessed were administered and followed-up for sample collection during infancy as described in section 4.3 (2) (b).

One of the limitations of the study was the challenge of whole blood collection from infants, and at most time points the quantity of whole blood collected from most infants was not always enough for CD15⁺ depletion. MDSC depletion was only performed when there was enough PBMC to perform the assays (section 4.3.2). Due to this, there were only a limited number of infants and their mothers at most time points for which depletion was performed and further permitting MDSC non-depleted versus MDSC depleted match paired analyses to

be performed (section 4.3.2). In this Appendix, unmatched analyses between MDSC non-depleted versus MDSC depleted samples in all participants enrolled and from who whole blood samples were collected are presented.

(i) Hep B induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in infants

As shown in Figure M1(i), there were no significant differences in either, (a) at the individual time points between groups in whole PBMC and without MDSC in infants at 10 and 14 weeks ($p=0.9789$ and $p=0.7255$ respectively); at 6 and 9 months ($p=0.066$ and $p=0.279$ respectively) and at 1 year of age ($p=0.512$), or (b) in the grouped analysis in whole PBMC and without MDSC at 10 and 14 weeks (median of CD15 non-depleted=0.330% versus CD15 depleted=0.180%; $p=0.718$) and at 6, 9 months and 1 year (median of CD15 non-depleted=0.170% versus CD15 depleted=0.587%; $p=0.481$).

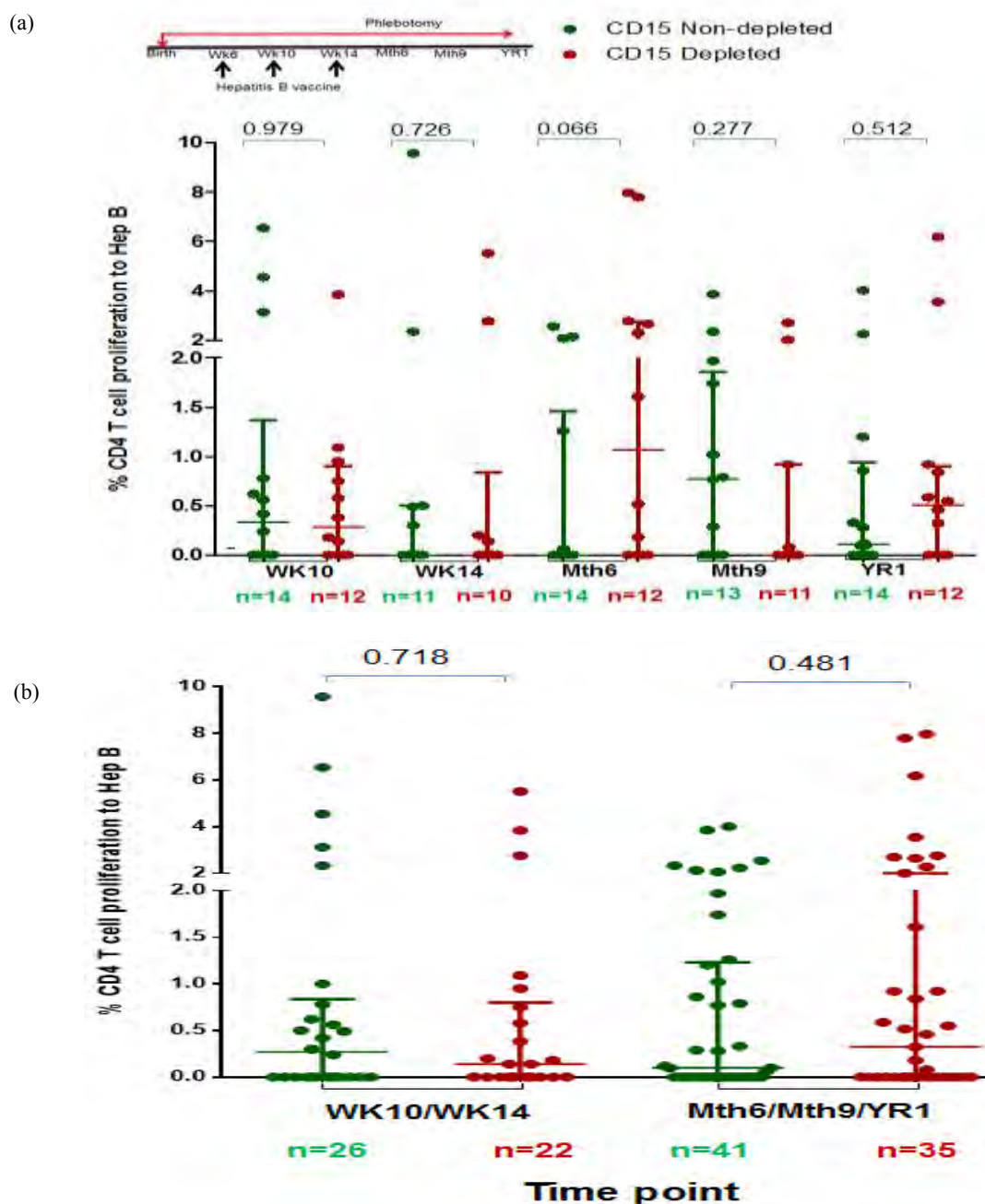


Figure M1 (i): $CD4^+$ T cell proliferative responses to Hep B in infants in whole PBMC versus without MDSC. (a) In the individual time point analysis, $CD4^+$ T cells proliferative responses to Hep B were measured in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red) at 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and (b) in the grouped analysis, at 10 and 14 weeks (after first and second doses of Hep B vaccination), at 6 and 9 months and one year of age (after last dose of Hep B vaccination). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

(ii) Hep B induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in mothers

In mothers, the CD4⁺ T cell proliferative responses to Hep B were evaluated in whole PBMC and without MDSC at delivery and again at one year postpartum. As shown in **Figure M1(ii)**, there was no significant difference at delivery (median of CD15 non-depleted=0.160% versus CD15 depleted=0.340%, $p=0.643$) and at one year postpartum (median of CD15 non-depleted=0.410% versus CD15 depleted=0.051%; $p=0.652$).

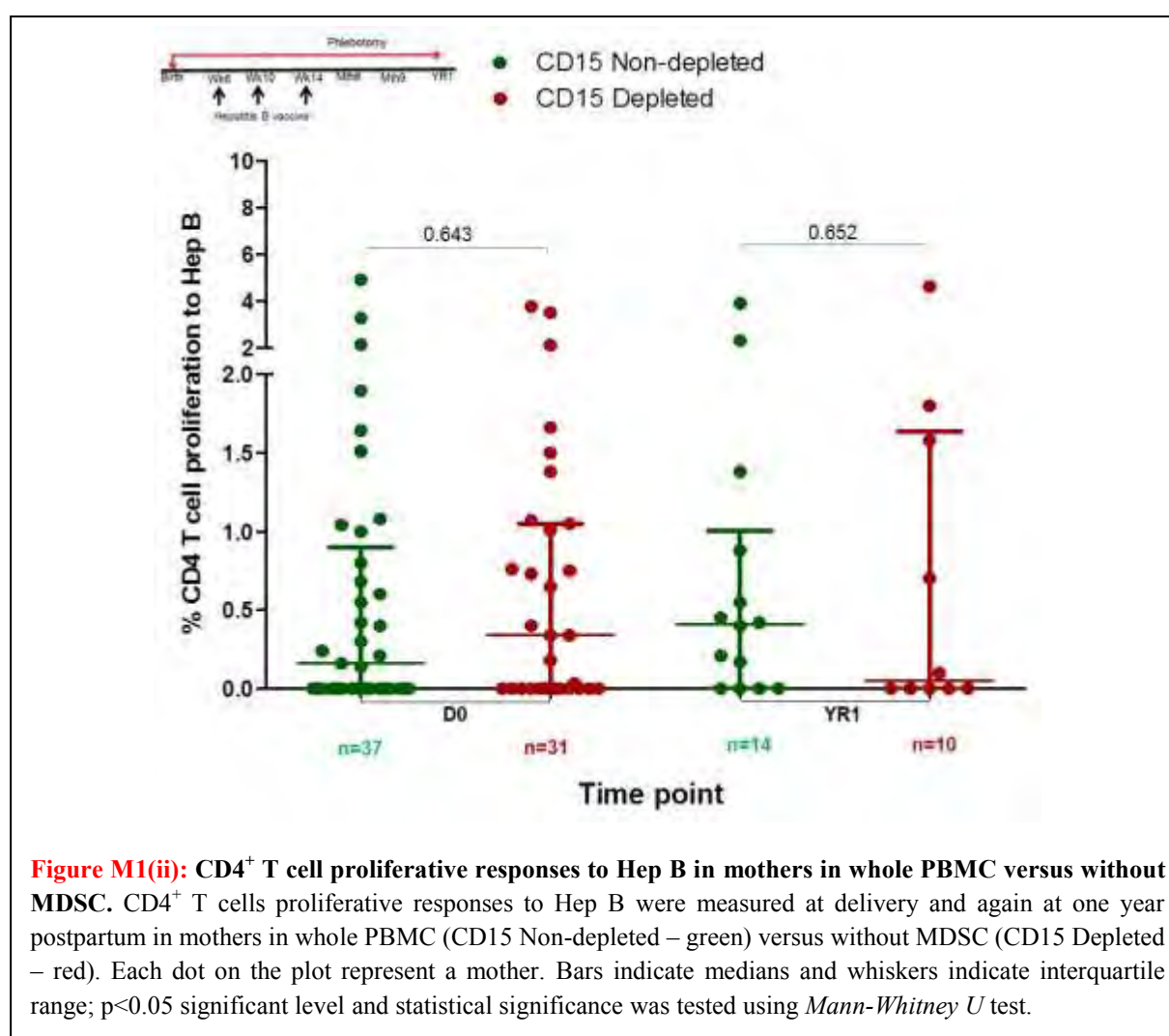
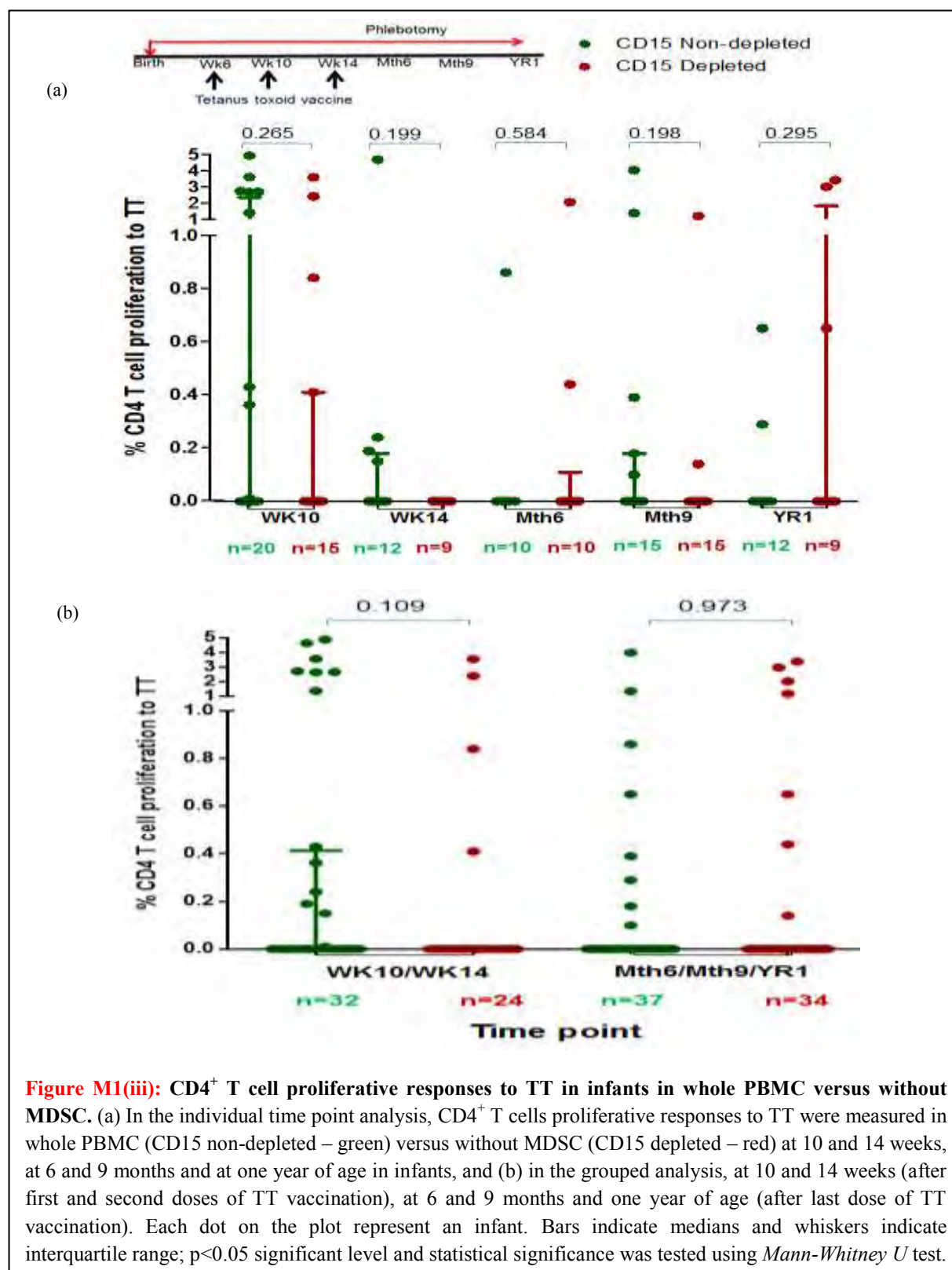


Figure M1(ii): CD4⁺ T cell proliferative responses to Hep B in mothers in whole PBMC versus without MDSC. CD4⁺ T cells proliferative responses to Hep B were measured at delivery and again at one year postpartum in mothers in whole PBMC (CD15 Non-depleted – green) versus without MDSC (CD15 Depleted – red). Each dot on the plot represent a mother. Bars indicate medians and whiskers indicate interquartile range; $p<0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

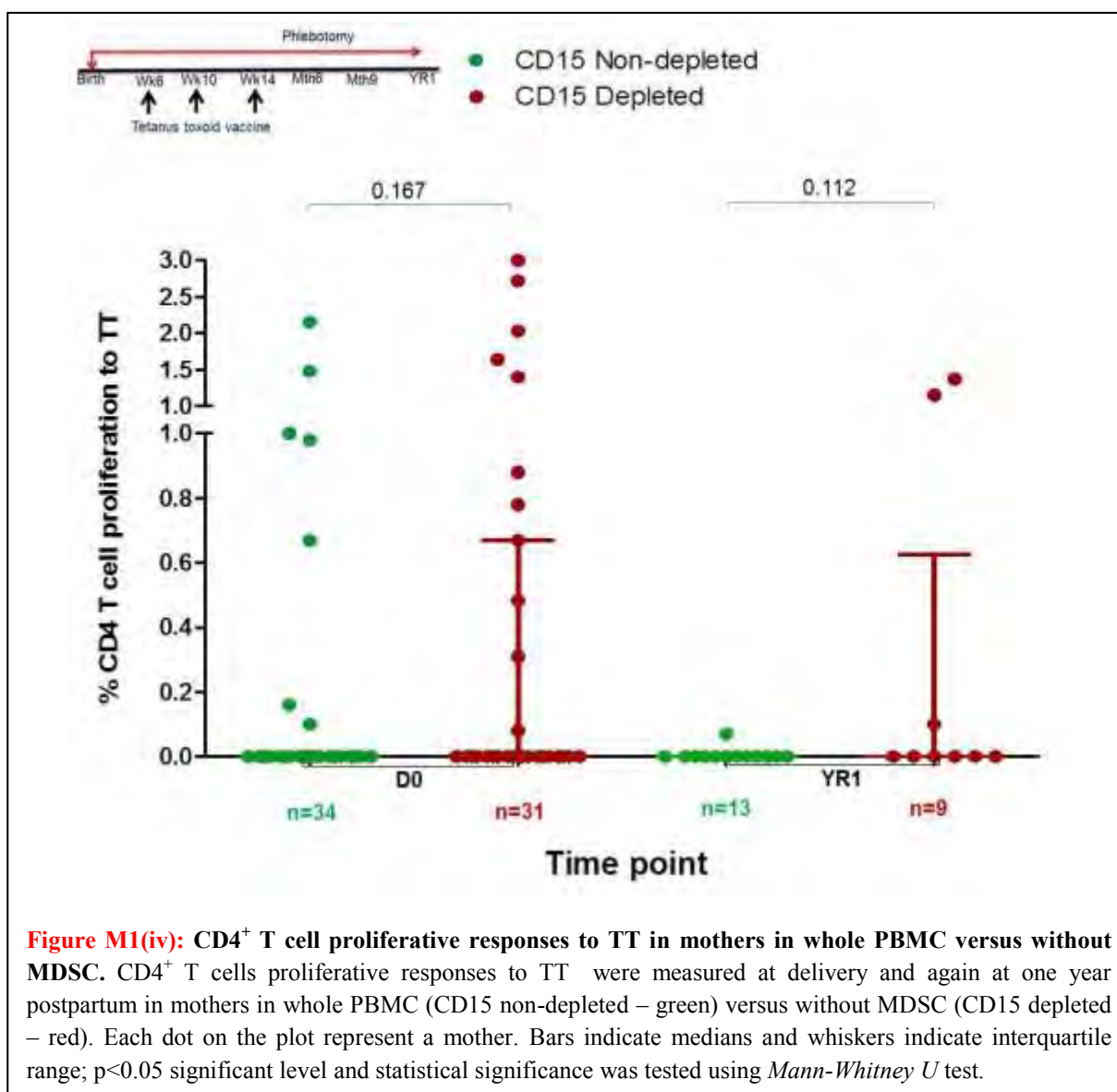
(iii) TT induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in infants

The CD4⁺ T cell proliferative responses to TT vaccination were evaluated in whole PBMC and without MDSC in infants at all time points post TT vaccination (Figure M1(iii)). We compared (a) the individual time points between groups in whole PBMC and without MDSC in infants. There was no significant difference at 10 and 14 weeks ($p=0.265$ and $p=0.199$ respectively), at 6 and 9 months ($p=0.584$ and $p=0.198$ respectively) and at one year ($p=0.295$); or (b) in the grouped analysis in T cell proliferation in whole PBMC and without MDSC there was no significant difference at 10 and 14 weeks ($p=0.109$) and at 6, 9 months and one year ($p=0.973$).



(iv) TT induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in mothers

In mothers, the CD4⁺ T cell proliferative responses to TT were evaluated in whole PBMC and without MDSC at delivery and again at one year postpartum. As shown in **Figure M1(iv)**, there was no significant difference at delivery (median of CD15 non-depleted=0%, IQR=0-0 versus CD15 depleted=0%, IQR=0-0.670 and $p=0.167$) and at one year postpartum (median of CD15 non-depleted=0%, IQR=0-0 versus CD15 depleted=0%, IQR=0-0.626 and $p=0.112$).



(v) PPD induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in infants

The CD4⁺ T cell proliferative responses to *Mycobacterium bovis* BCG vaccination were evaluated in whole PBMC and without MDSC in infants at birth, at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age, by measuring responses to PPD.

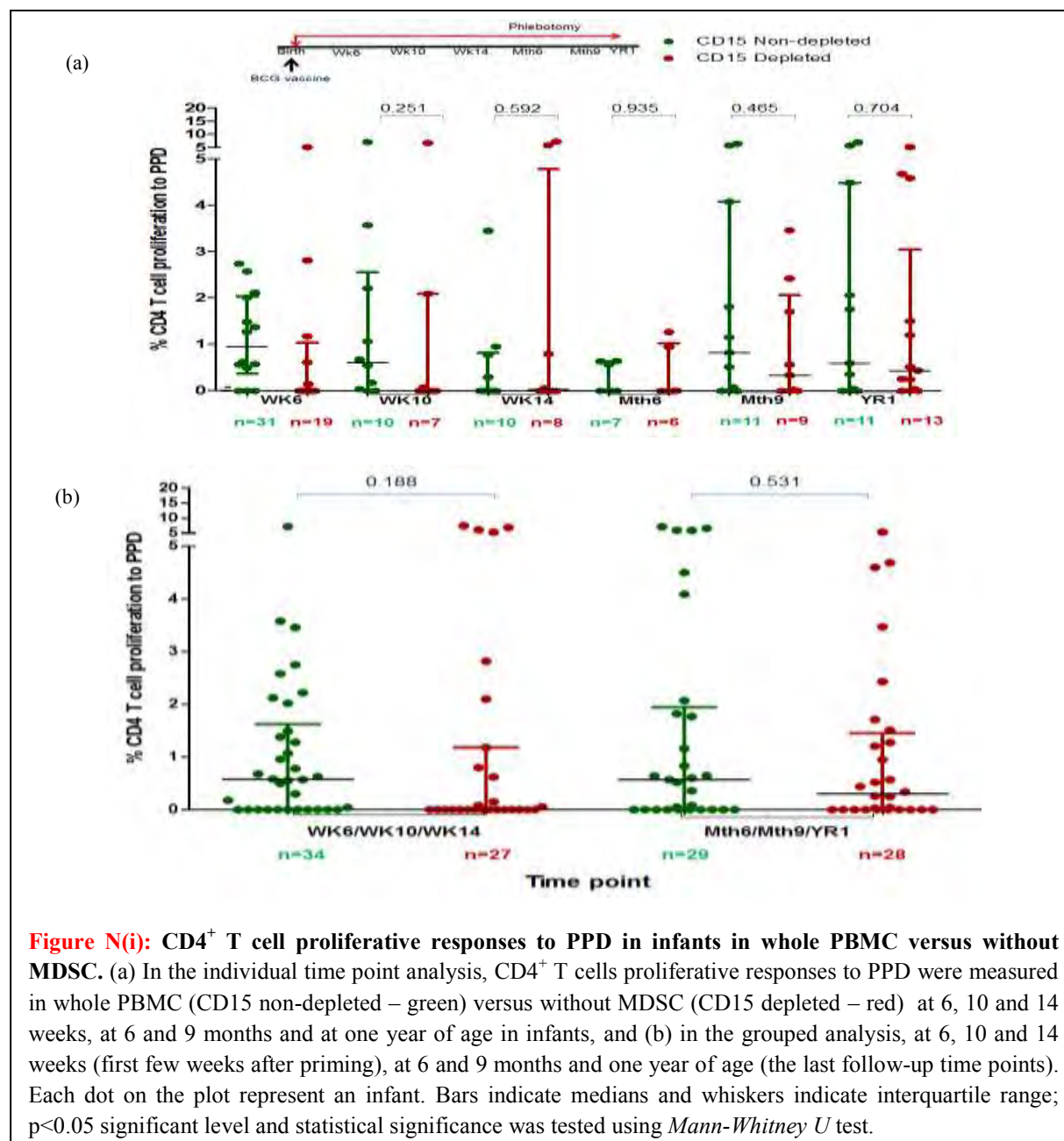


Figure N(i): CD4⁺ T cell proliferative responses to PPD in infants in whole PBMC versus without MDSC. (a) In the individual time point analysis, CD4⁺ T cells proliferative responses to PPD were measured in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red) at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and (b) in the grouped analysis, at 6, 10 and 14 weeks (first few weeks after priming), at 6 and 9 months and one year of age (the last follow-up time points). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; p<0.05 significant level and statistical significance was tested using *Mann-Whitney U* test.

There were no significant differences in either, (a) at the individual time points at 6, 10 and 14 weeks ($p=0.1247$, $p=0.2513$ and $p=0.5916$ respectively), at 6 and 9 months ($p=0.935$ and $p=0.455$ respectively) and at one year ($p=0.704$), or (b) in the grouped analysis with and without MDSC at 6, 10 and 14 weeks (median of CD15 non-depleted=0.684% versus CD15 depleted=0.15%; $p=0.188$) and at 6, 9 months and one year (median of CD15 non-depleted=0.830% versus CD15 depleted=0.480%; $p=0.531$) (Figure N(i)).

(vi) PPD induced $CD4^+$ T cell proliferation in whole PBMC versus after MDSC depletion in mothers

In mothers, $CD4^+$ T cell proliferative responses to PPD were evaluated in whole PBMC and without MDSC at delivery and again at one year postpartum. As shown in Figure N(ii), there was no significant difference at delivery (median of CD15 non-depleted=0.100% versus CD15 depleted=0.00% and $p=0.241$) and at one year postpartum (median of CD15 non-depleted=0.370% versus CD15 depleted=0.200% and $p=0.877$).

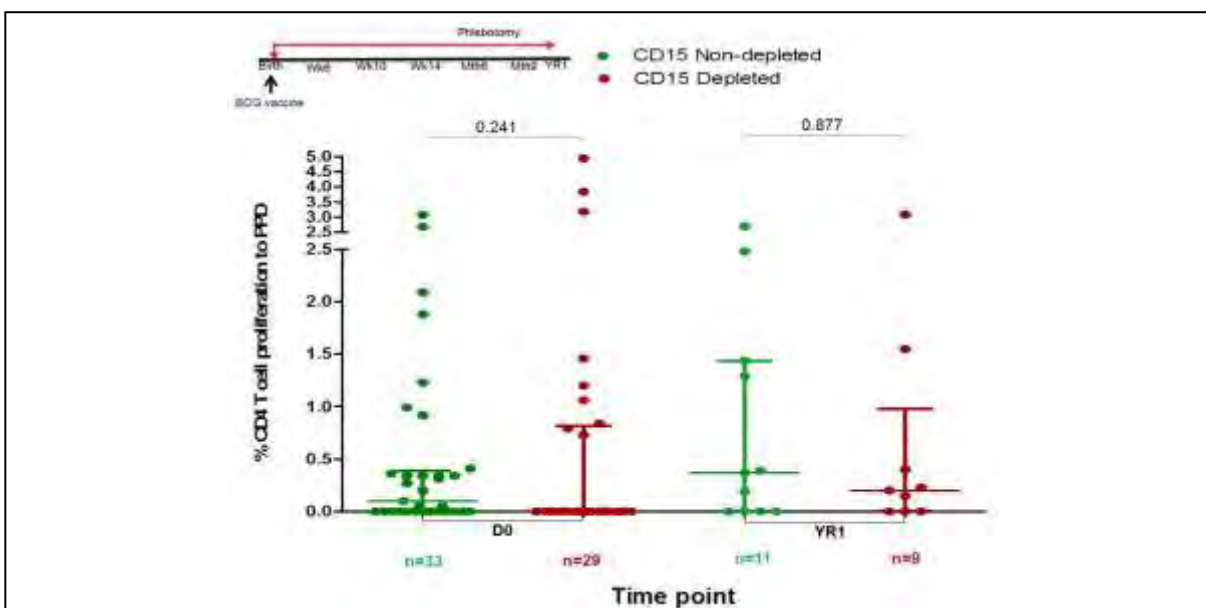


Figure N(ii): $CD4^+$ T cell proliferative responses to PPD in mothers in whole PBMC versus without MDSC. $CD4^+$ T cells proliferative responses to PPD were measured at delivery and again at one year postpartum in mothers in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red). Each dot on the plot represent a mother. Bars indicate medians and whiskers indicate interquartile range; $p<0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

(vii) Dynabead induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in infants

Anti-CD3CD28 (Dynabead) was employed as a positive control in this study, as it has been shown to be strong T cell activator acting directly on T cell receptors and does not require feeder cells (antigen presenting cells) or antigens (Li *et al.*, 2010)(Oviedo-Orta *et al.*, 2010). To investigate the influence of MDSC on T cell proliferation to strong, non-specific stimulation, we evaluated CD4⁺ T cell proliferative responses to α -CD3CD28 (Dynabead) stimulation in whole PBMC and without MDSC in infants at birth, at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age (Figure O). Proliferation at all time points was robust. There was no significant difference at birth (median of CD15 Non-depleted=80.73% versus CD15 Depleted=67.40% and $p=0.073$); at 6, 10 and 14 weeks ($p=0.177$, $p=0.299$ and $p=0.111$ respectively); at 6 and 9 months ($p=0.738$ and $p=0.159$ respectively) and at one year ($p=0.763$). In general, proliferation decreased post depletion.

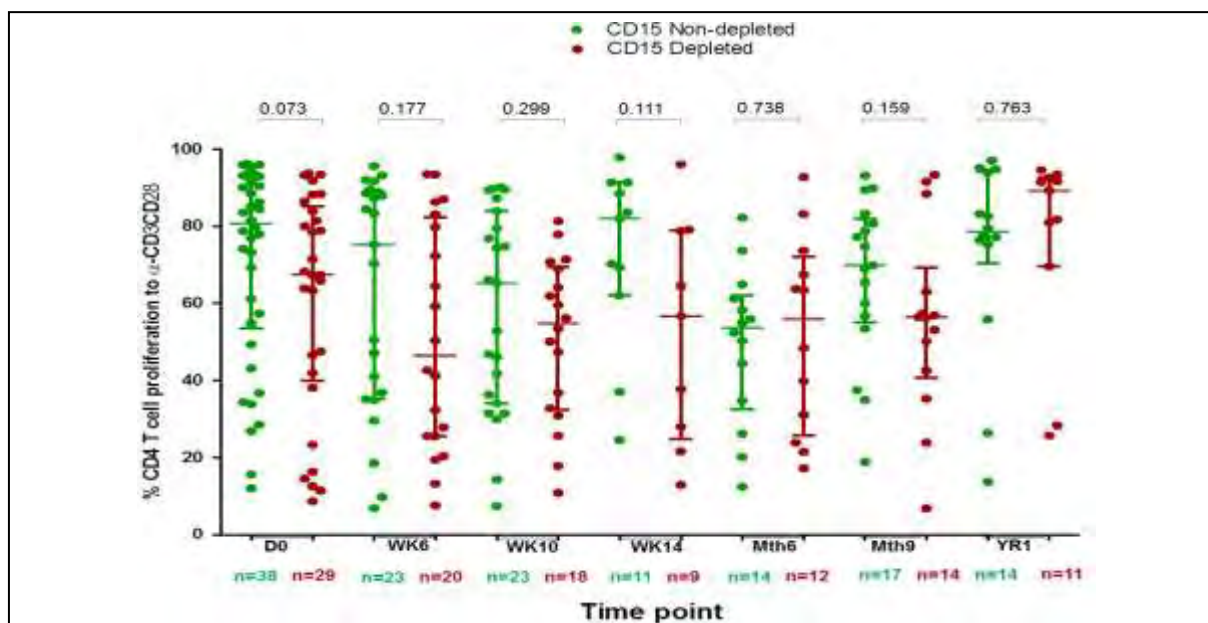
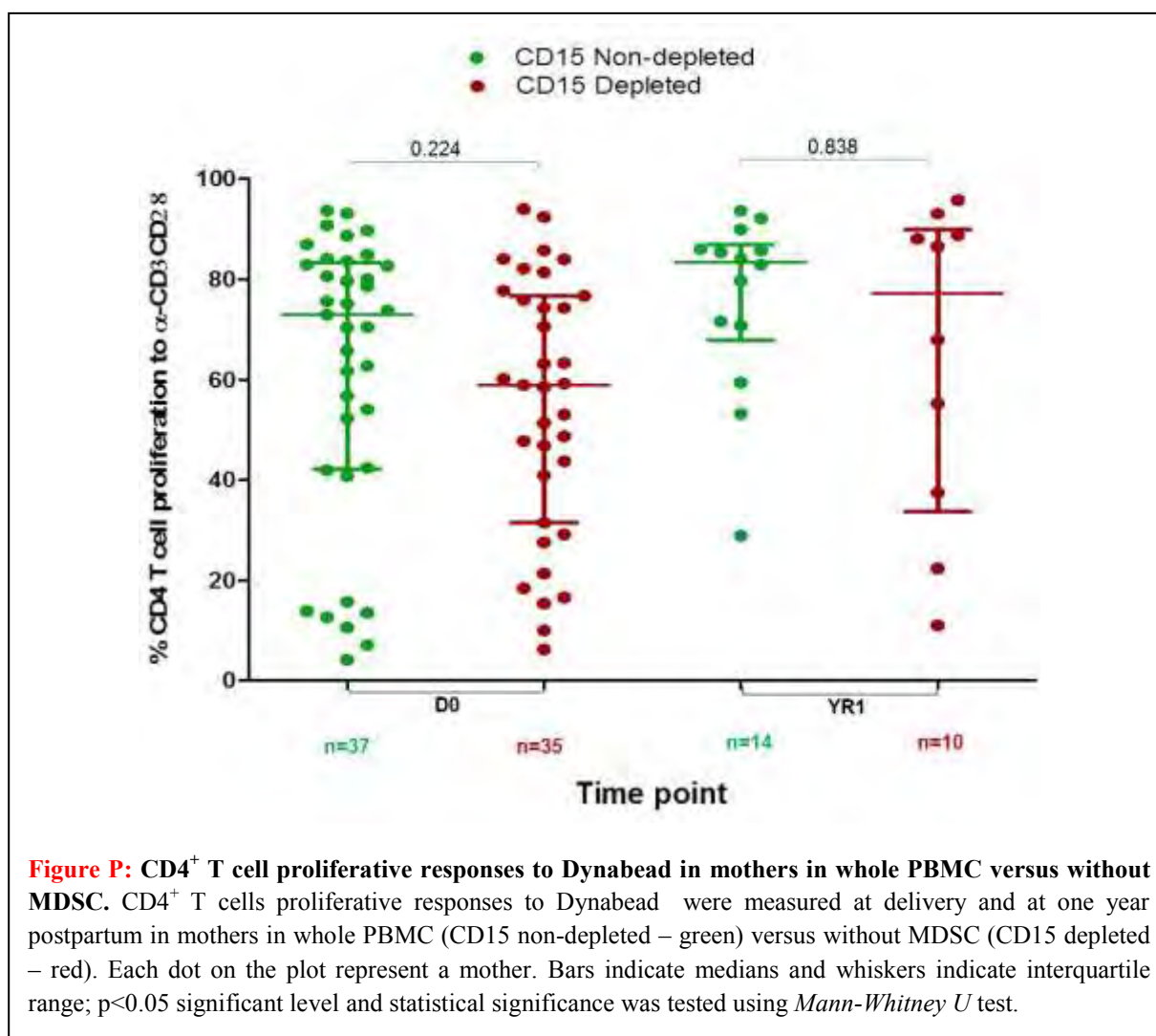


Figure O: CD4⁺ T cell proliferative responses to Dynabead in infants in whole PBMC versus without MDSC. CD4⁺ T cells proliferative responses to Dynabead were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p<0.05$ significant level and statistical significance was tested using Mann-Whitney U test.

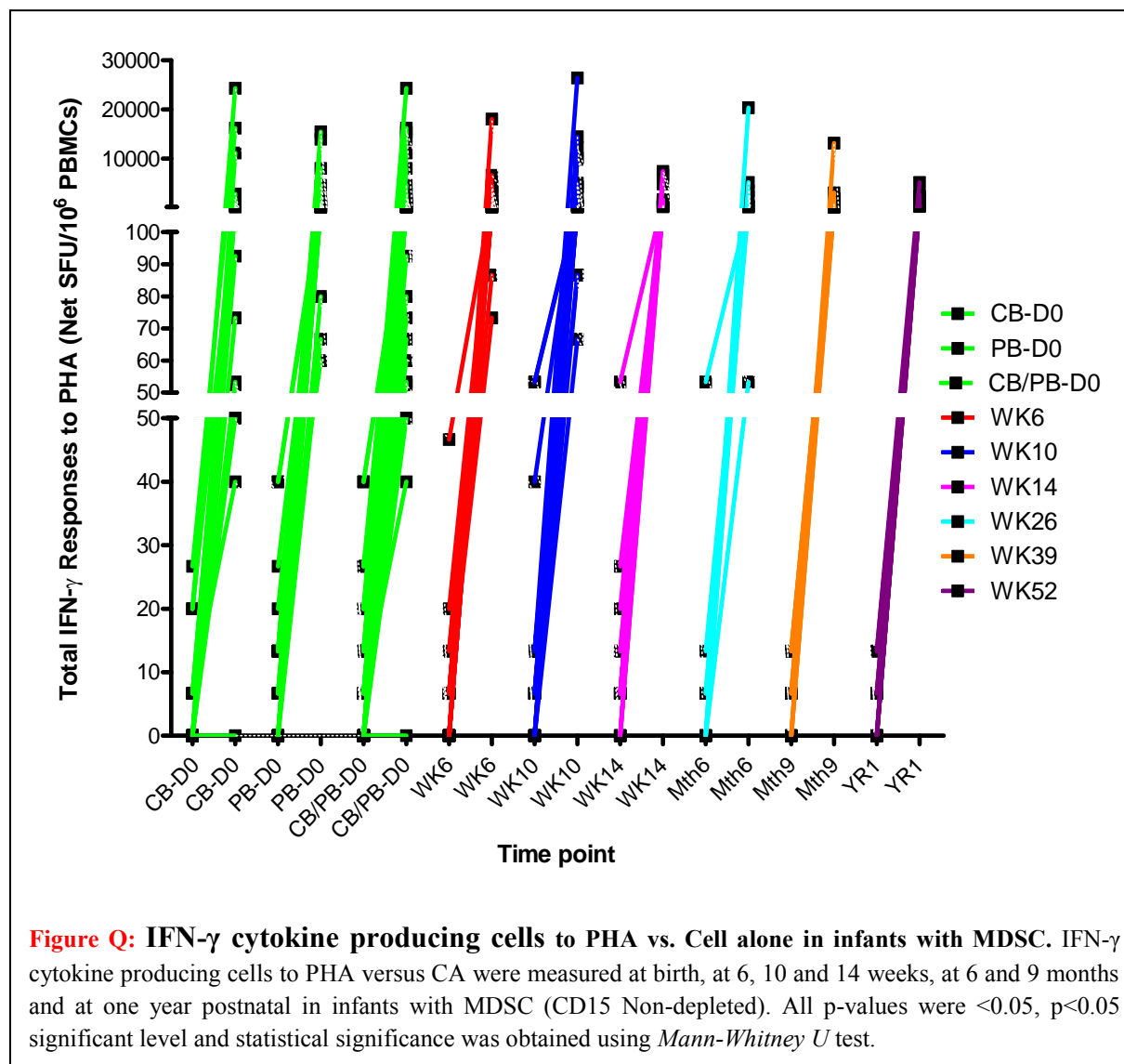
4.3.2.20 Dynabead induced CD4⁺ T cell proliferation in whole PBMC versus after MDSC depletion in mothers

In mothers, the CD4⁺ T cell proliferative responses to Dynabead were evaluated in whole PBMC and without MDSC at delivery and at one year postpartum. As shown in **Figure P**, there was no significant difference at delivery (median of CD15 non-depleted=72.91% versus CD15 depleted=58.88%; $p=0.224$) and at one year postpartum (median of CD15 non-depleted=83.45% versus CD15 depleted=77.23%; $p=0.838$).

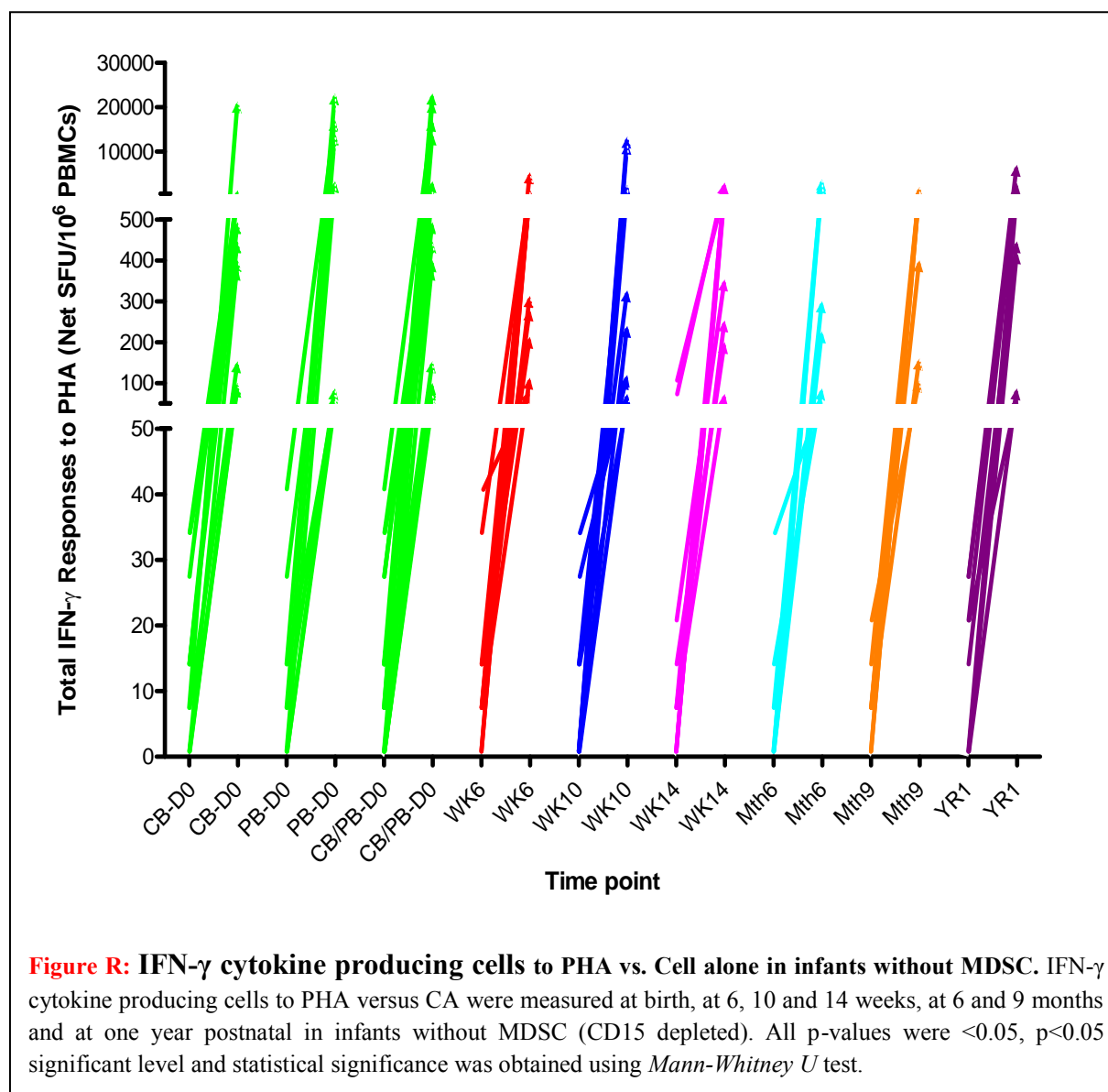


Appendix 5.1: IFN- γ ELISpot assay validity

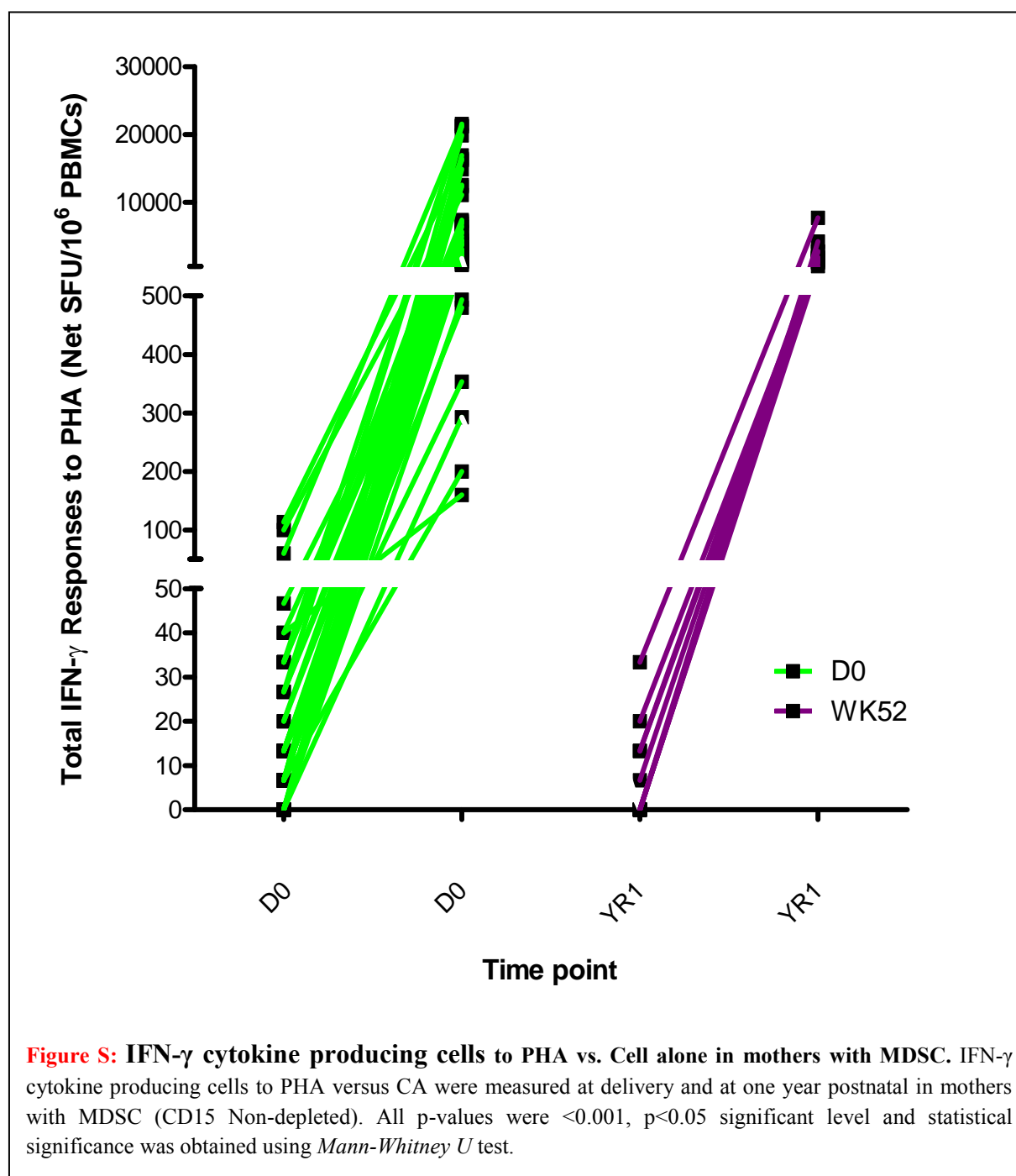
(i) Phytohaemagglutinin (PHA) causes IFN- γ production in infants vs. Cells alone (CA) with MDSC



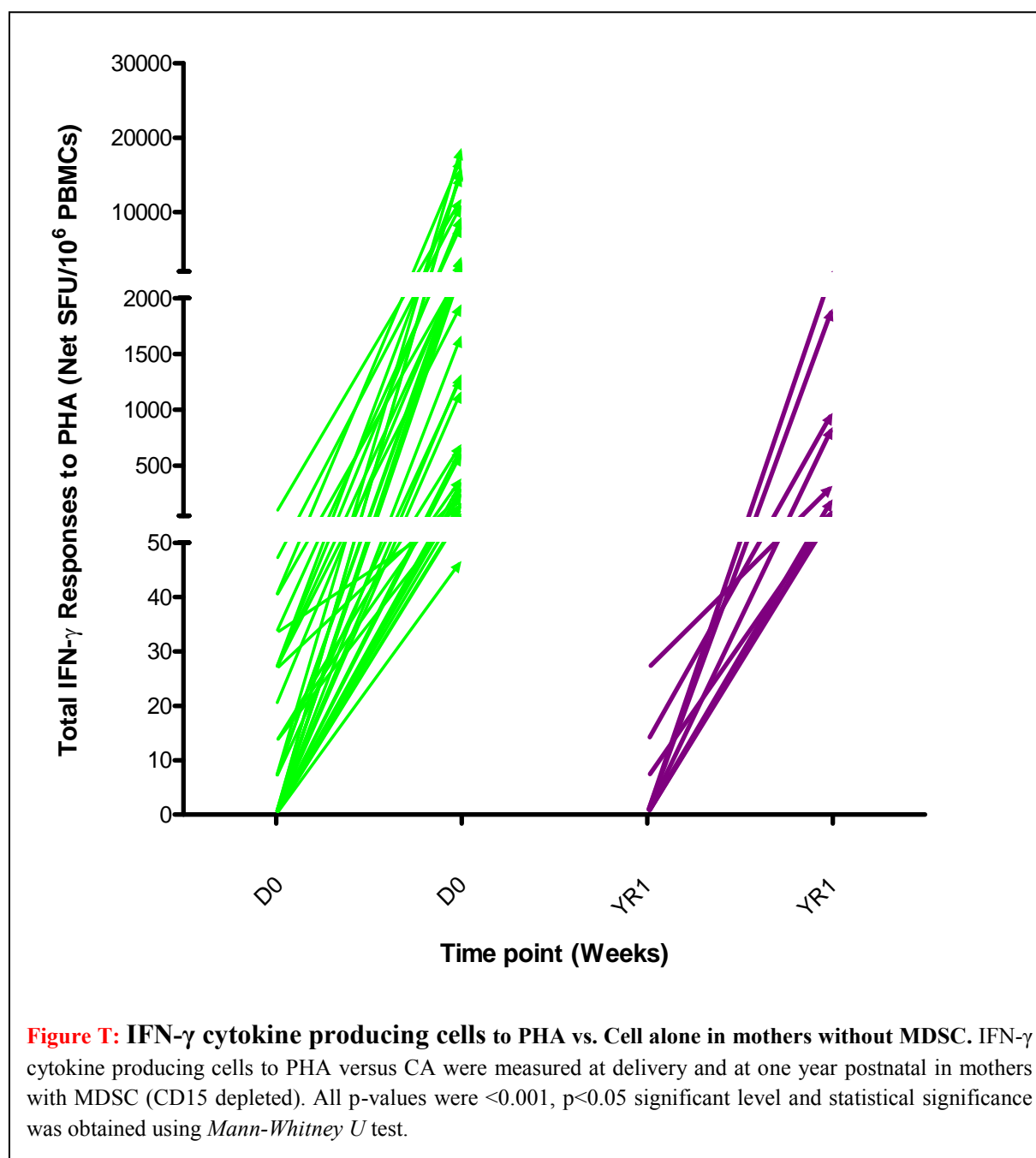
(ii) PHA causes IFN- γ production in infants vs. Cells alone (CA) without MDSC



(iii) PHA causes IFN- γ production in mothers vs. Cells alone (CA) with MDSC



(iv) PHA causes IFN- γ production in mothers vs. Cells alone (CA) without MDSC



Appendix 5.2: IFN- γ cytokine producing cells to vaccines in infants and mothers through time

Generally, vaccine responses varied in the different antigens through time. Some responses to vaccines were stronger and some low. Also, responses varied between infants and their mothers. Below is the general dynamic in vaccine responses (median counts of IFN- γ cytokine producing cells) to the different antigens in infants and mothers through time regardless of MDSC.

The number of infants excluded due to invalid ELISpot assay (IFN- γ SFU/million cells [PHA] > IFN- γ SFU/million cells (Median + 3MAD)[Media])

In total 70 mother-infant pairs were recruited at delivery, 39 followed up at 6 weeks, 32 at 10 weeks, 30 at 14 weeks, 20 at 6 months, 15 at 9 months and lastly 14 infant-mother pairs completed the study at one year postpartum (section 2.2). Experiments were valid when the number of IFN- γ Spot Forming Units (SFU)/million cells in response to PHA was greater than the median plus 3 times the MAD of the negative control (i.e. Media) IFN- γ SFU/million cells, or the sample was excluded; *i.e.* IFN- γ SFU/million cells [PHA] > IFN- γ SFU/million cells (Median + 3MAD)[Media] where MAD is the Median Absolute Deviation Section 5.2.2. Table A shows the proportion of samples excluded from the analyses.

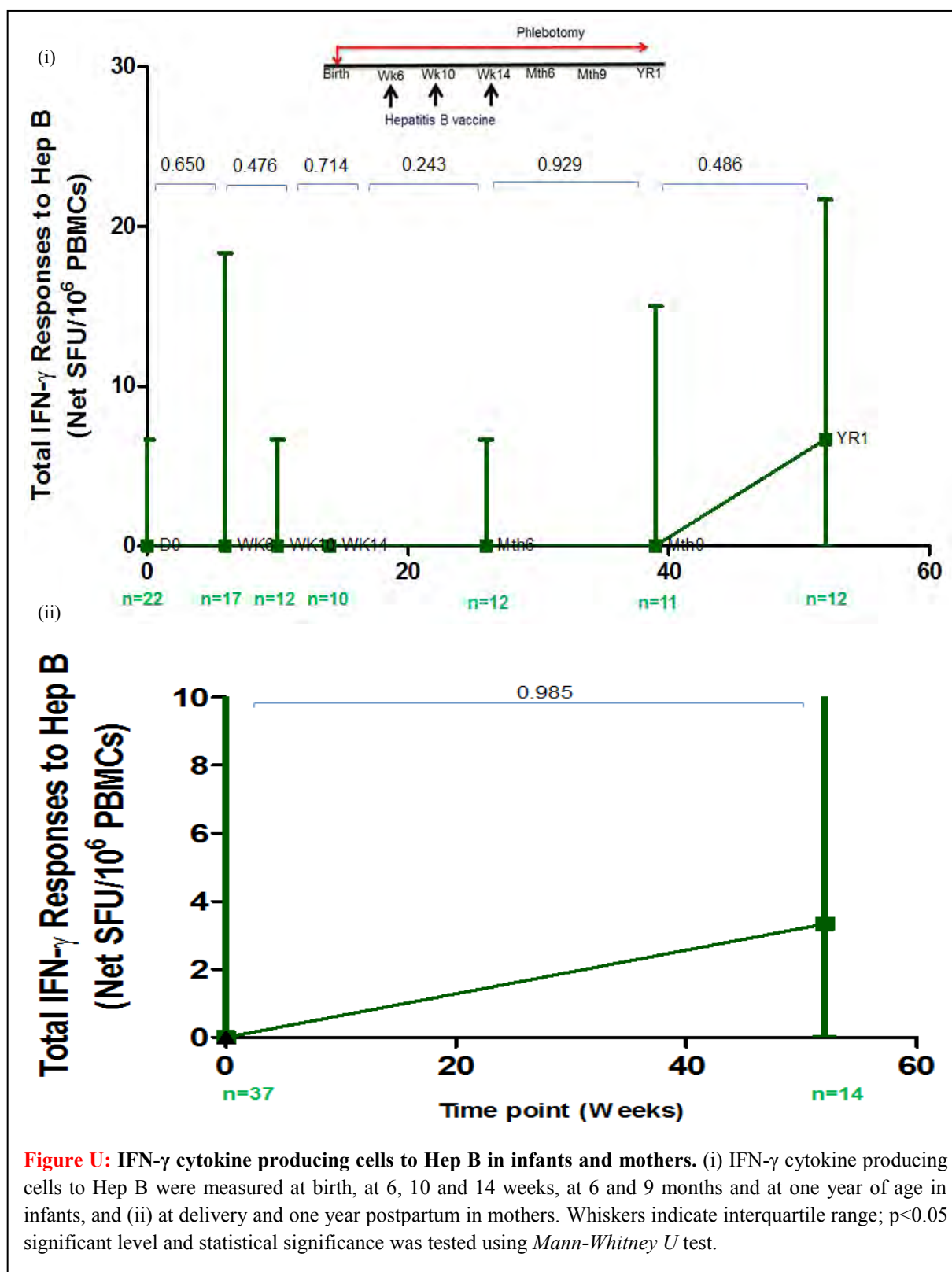
Table A: The number of infants excluded from ELISpot analyses. Time point include the different time points post vaccination i.e. WK6 post *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), WK10 post *Bordetella pertussis* (BP) and tetanus toxoid (TT) and the further respective time points WK14, Mth6, Mth9 and one year.

Time point	WK6		WK10		WK14		Mth6		Mth9		YR1	
Condition	Non-depl.	Depl.	Non-depl.	Depl.	Non-depl.	Depl.	Non-depl.	Depl.	Non-depl.	Depl.	Non-depl.	Depl.
	6	6	6	1	7	1	6	6	8	4	1	1
	13	7	7	6	8	8	7	7	10	9	12	12
	26	8	8	7	18	18	8	8		10		
	29	9	9	8	20	22	9	14		20		
		13	19	9	22		11	18				
		20	20	12	27		14	20				

		26	23	18			18	22					
		29		19			22						
		35		20									
				22									
				23									
Total Excluded	#	4	9	7	11	6	4	8	7	2	4	2	2
Total recruited	#	39	39	32	32	30	30	20	20	15	15	14	14
Proportion excluded (%)		10.3	23.1	21.9	34.4	20.0	13.3	40.0	35.0	13.3	26.7	14.3	14.3

(i) IFN- γ cytokine producing cells to vaccines in infants and mothers through time after Hep B stimulation

Generally, Hep B responses were low at 10 and 14 weeks after priming at 6 weeks in infants and remained low till 6 months of age. The responses then peaked at 9 months to one year of age [Figure U(i)]. While in the mothers, Figure U(ii), Hep B responses increased from delivery to one year postpartum.



(ii) IFN- γ cytokine producing cells to vaccines in infants and mothers through time after TT stimulation

Generally, TT responses were low at birth before priming at 6 weeks of age, then peaked at 10 weeks before the second vaccine dose in infants and dropped at 14 weeks before the third vaccine dose. The responses then further remained low post the third vaccine dose at 14 weeks of age till 6, 9 months and one year of age [Figure V(i)]. In the mothers, Figure V(ii), TT responses dropped from delivery to one year postpartum.

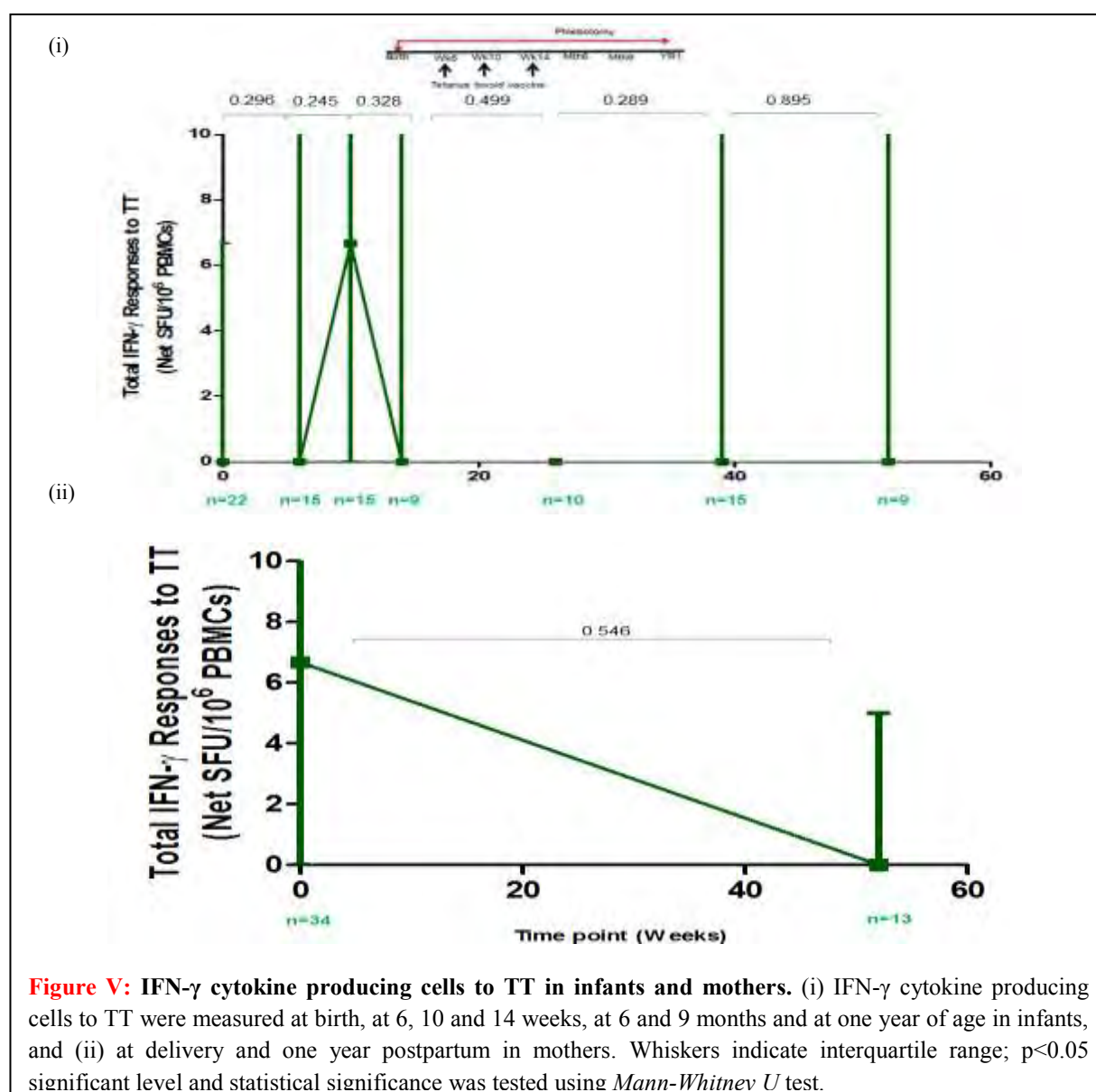


Figure V: IFN- γ cytokine producing cells to TT in infants and mothers. (i) IFN- γ cytokine producing cells to TT were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and (ii) at delivery and one year postpartum in mothers. Whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

(iii) IFN- γ cytokine producing cells to vaccines in infants and mothers through time after BP stimulation

Generally, BP responses were low before priming at 6 weeks of age, remained low till 10 weeks of age before the second vaccine dose. BP responses then peaked at 14 weeks and varied till one year of age [Figure W(i)]. In the mothers, Figure W(ii), BP responses remained low from delivery to one year postpartum.

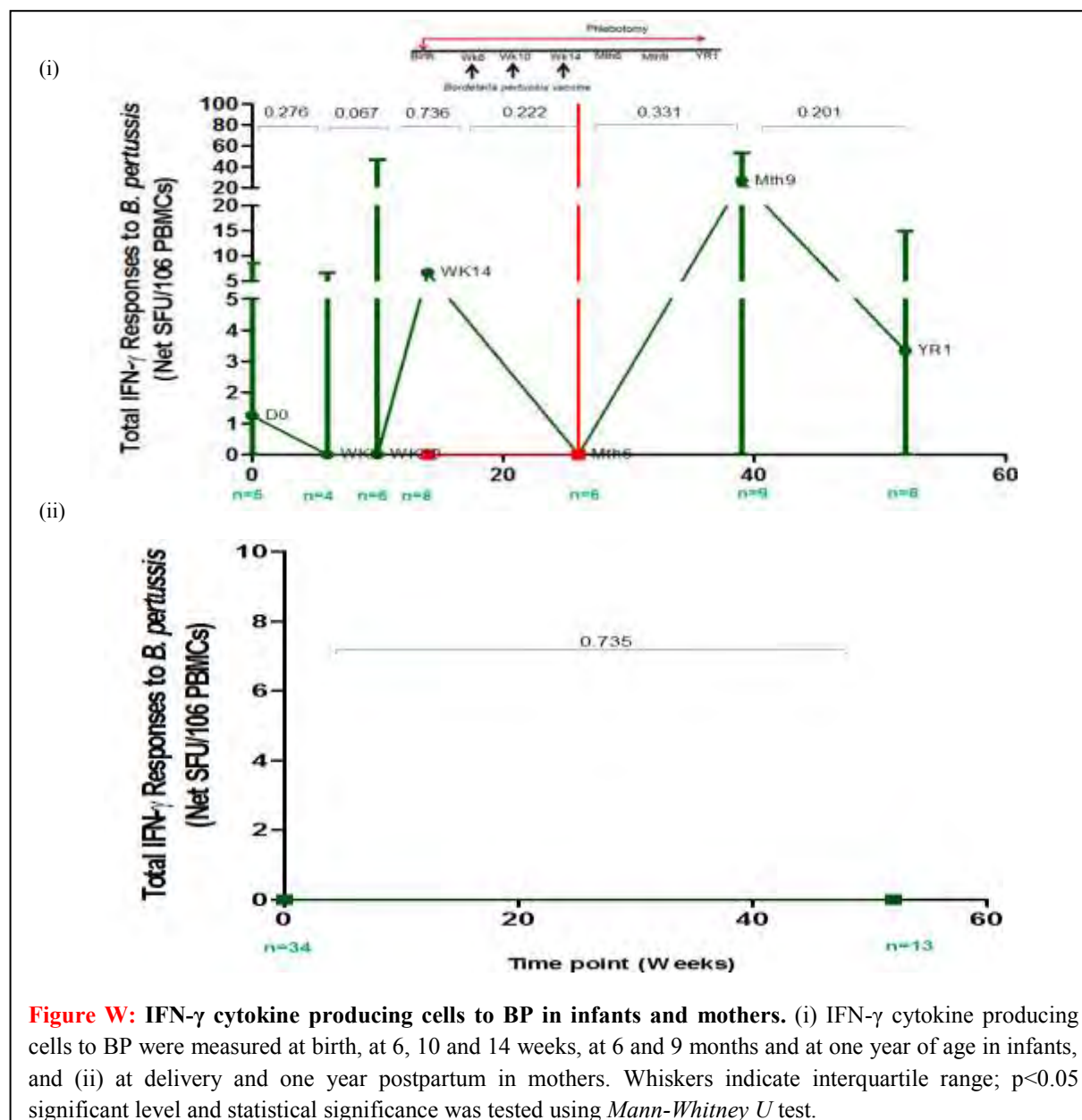


Figure W: IFN- γ cytokine producing cells to BP in infants and mothers. (i) IFN- γ cytokine producing cells to BP were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and (ii) at delivery and one year postpartum in mothers. Whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

(iv) IFN- γ cytokine producing cells to vaccines in infants and mothers through time after BCG stimulation

Generally, BCG responses peaked from birth to 6, 10 and 14 weeks after BCG vaccination at birth in infants and then dropped at 6 months of age. The responses then remained low from 6 months through 9 months to one year of age [Figure X(i)]. While in the mothers, Figure X(ii), BCG responses dropped from delivery to one year postpartum.

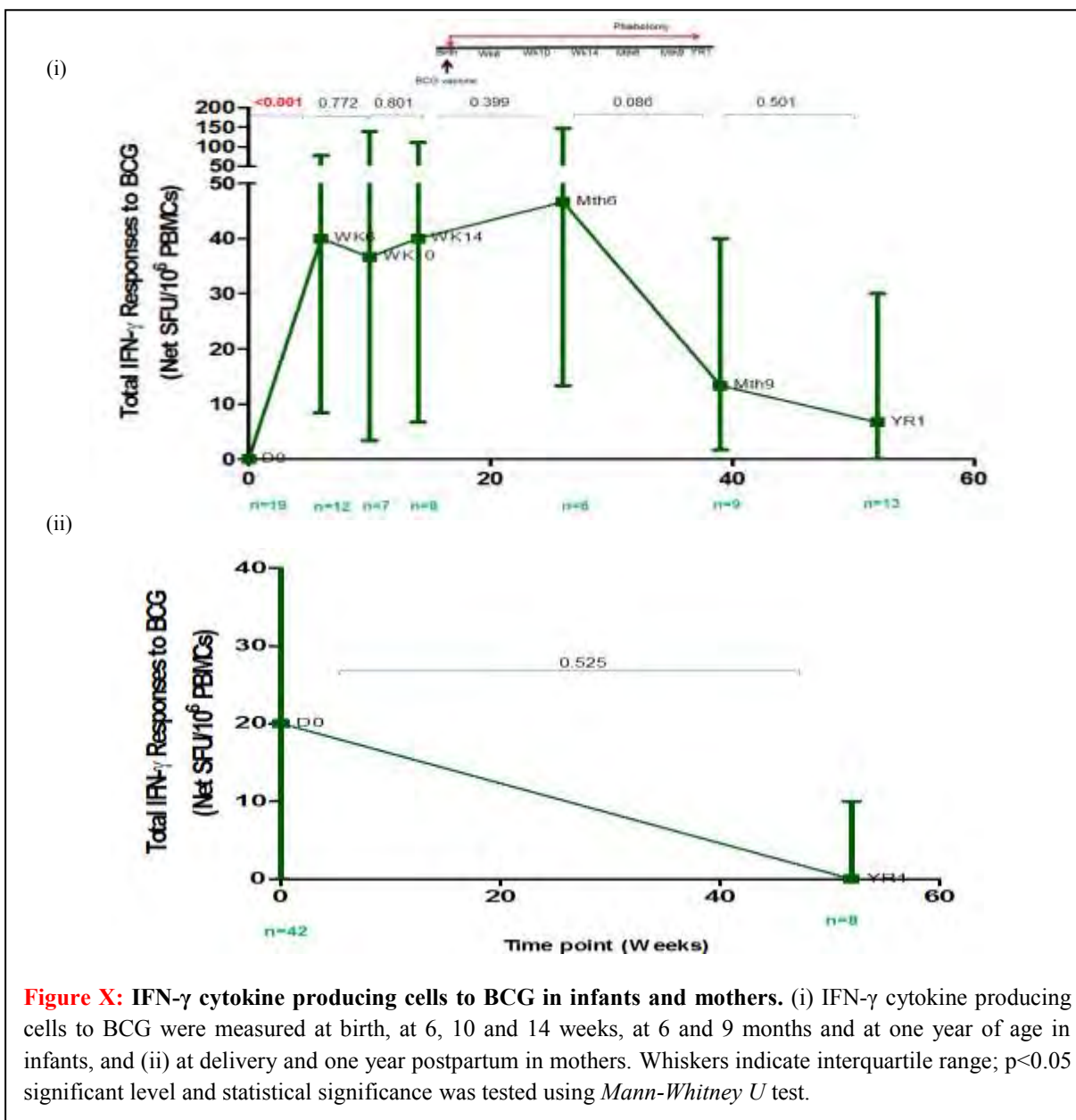


Figure X: IFN- γ cytokine producing cells to BCG in infants and mothers. (i) IFN- γ cytokine producing cells to BCG were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and (ii) at delivery and one year postpartum in mothers. Whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

(v) IFN- γ cytokine producing cells to Phytohaemagglutinin (PHA) in infants and mothers through time

Generally, PHA responses as expected were strong from birth through 6, 10 and 14 weeks in infants till 6 and 9 months of age. The responses increased with age. [Figure Y(i)]. While in the mothers, Figure Y(ii), PHA responses were high at deliver, but drop at one year postpartum.

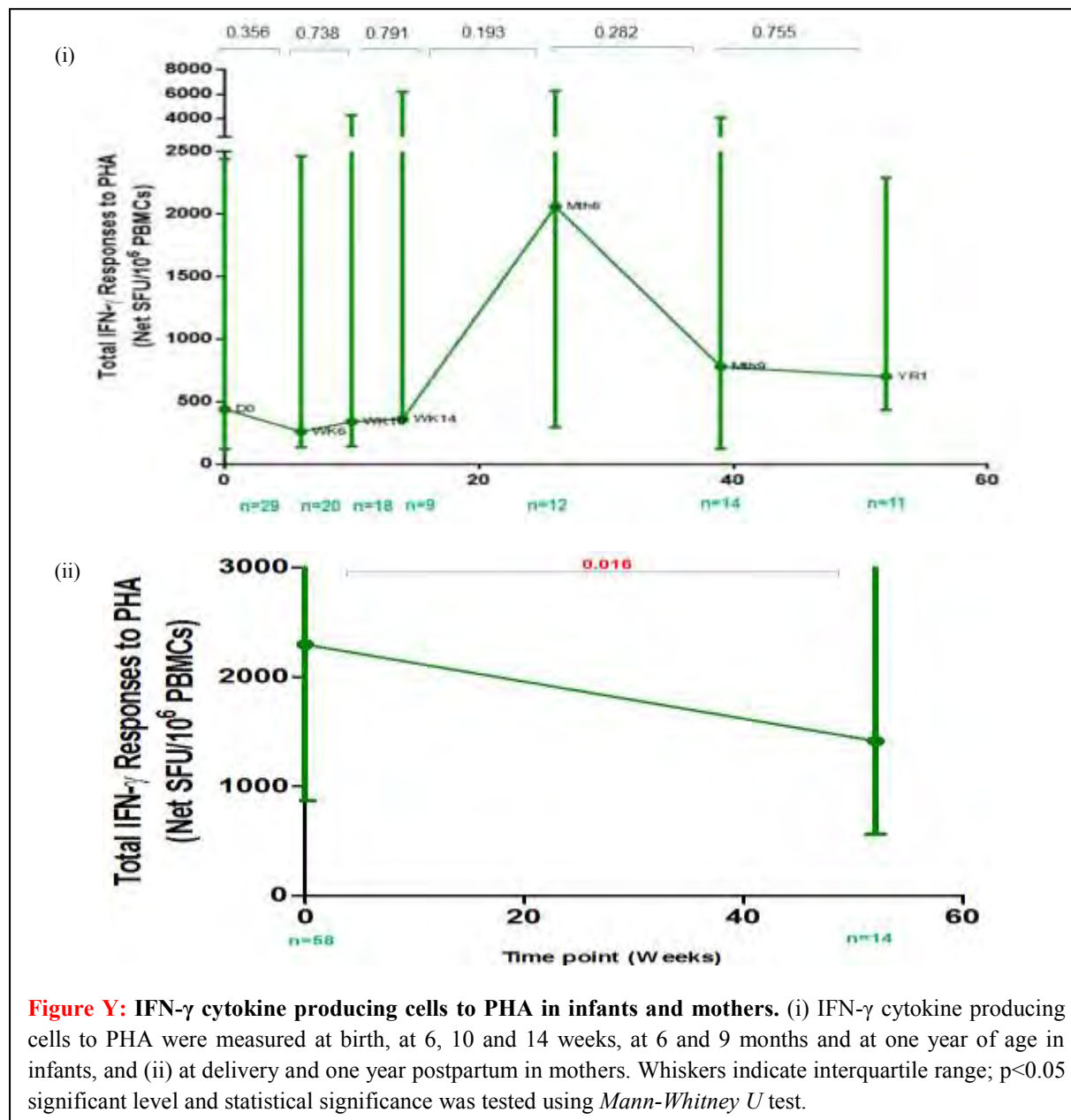


Figure Y: IFN- γ cytokine producing cells to PHA in infants and mothers. (i) IFN- γ cytokine producing cells to PHA were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and (ii) at delivery and one year postpartum in mothers. Whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

Appendix 5.3: Effect of MDSC depletion on IFN- γ production to vaccines and antigens in all infants and mothers enrolled from who whole blood samples were collected

The effect of MDSC on IFN- γ cytokine producing cells to BCG, TT, BP, and Hep B vaccinations, and to PHA, in whole CBMC and/or PBMC were evaluated in infants and mothers in the presence of MDSC and after MDSC depletion (Figure 4.1). Since MDSC highly express CD15, as earlier described in section 4.2.1, CD15⁺ expressing cells were magnetically depleted from a proportion of whole CBMC and/or PBMC. The effect of MDSC on cells producing IFN- γ to vaccines and antigens was then measured by performing the IFN- γ ELISpot assay on CD15 depleted cells (absence of MDSC) and whole CBMC and/or PBMC (presence of MDSC) (Figure 4.2). In the MDSC depleted sample group, only samples with depletion purity of 90% or more were included in the analyses (Appendix 4.3).

As described in section 5.3 (2), IFN- γ cytokine producing cells were measured in infants at all individual time points post vaccination (i.e. at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age), and in mothers, the cells producing IFN- γ to vaccines and antigens were measured at delivery and again at one year postpartum. We also evaluated the ontogeny of the effect of MDSC on IFN- γ cytokine producing cells to vaccines and antigens in a grouped time points analyses, based on the time points the different vaccines assessed were administered and followed-up for sample collection during infancy as described in section 5.3 (2) (b).

Generally, vaccine responses to the different antigens varied through time. Also, responses varied between infants and their mothers. Regardless of MDSC, in Appendix 5.2, is the general dynamic in vaccine responses (median counts of IFN- γ cytokine producing cells) to the different antigens in infants and mothers through time.

(i) BCG induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in infants

The cells producing IFN- γ to BCG vaccination were evaluated in whole PBMC and without MDSC in infants at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age. As shown in **Figure Z** there were no significant differences at most time points. However, IFN- γ cytokine producing cells to BCG lasted till 9 months. (a) At the individual time points there were no

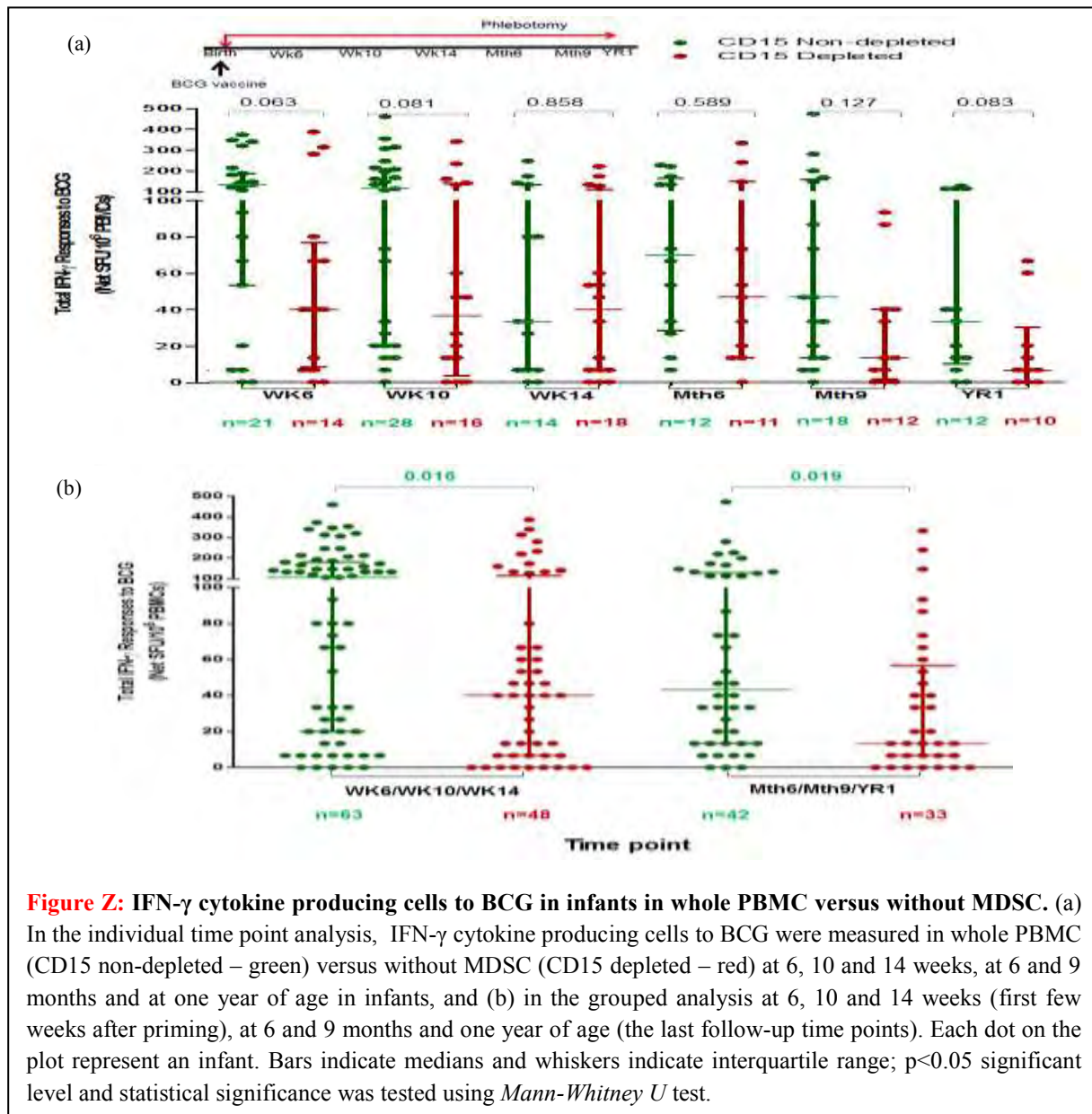
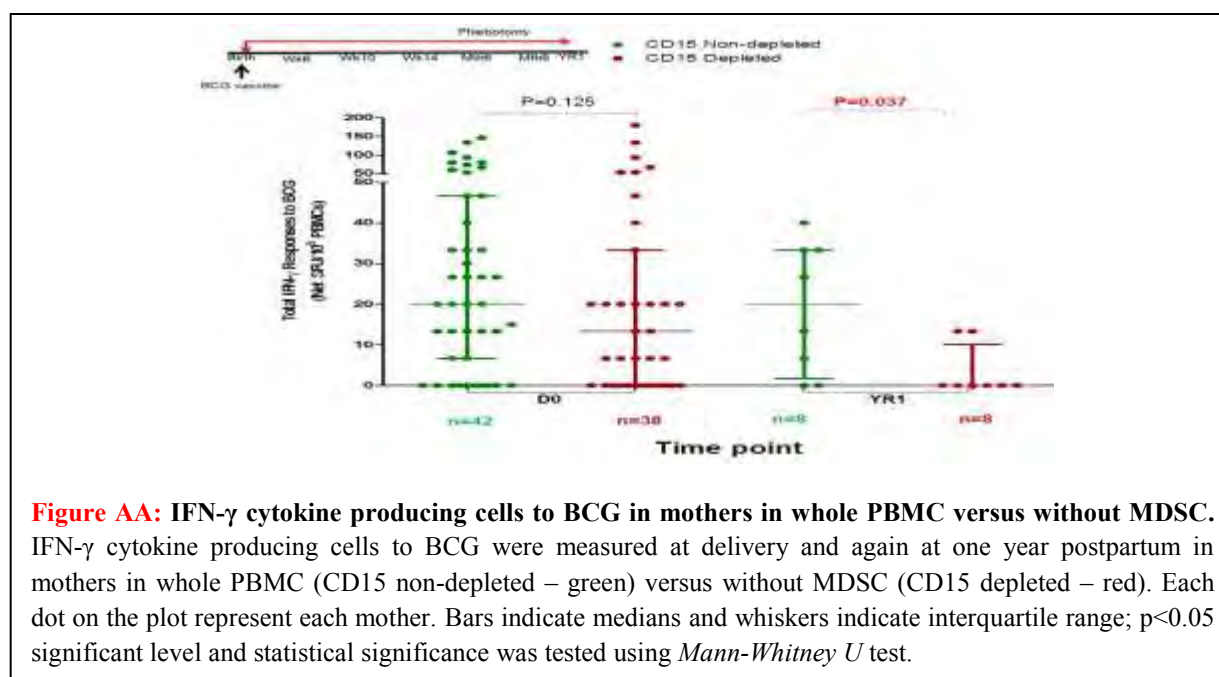


Figure Z: IFN- γ cytokine producing cells to BCG in infants in whole PBMC versus without MDSC. (a) In the individual time point analysis, IFN- γ cytokine producing cells to BCG were measured in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red) at 6, 10 and 14 weeks, at 6 and 9 months and at one year of age in infants, and (b) in the grouped analysis at 6, 10 and 14 weeks (first few weeks after priming), at 6 and 9 months and one year of age (the last follow-up time points). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

significant differences at 6, 10 and 14 weeks ($p=0.063$, $p=0.081$ and $p=0.858$ respectively), at 6 and 9 months ($p=0.589$ and $p=0.127$ respectively) and at one year ($p=0.083$). However, (b) in the grouped analysis IFN- γ cytokine producing cells to BCG were significantly higher in whole PBMC compared to after MDSC depletion at 6, 10 and 14 weeks (median of CD15 non-depleted=103.330 Net SFU/ 10^6 PBMC versus CD15 depleted=39.670 Net SFU/ 10^6 PBMC; $p=0.016$) and at 6, 9 months and 1 year (median of CD15 non-depleted=43.670 Net SFU/ 10^6 PBMC versus CD15 depleted=15.330 Net SFU/ 10^6 PBMC; $p=0.019$) (Figure Z).

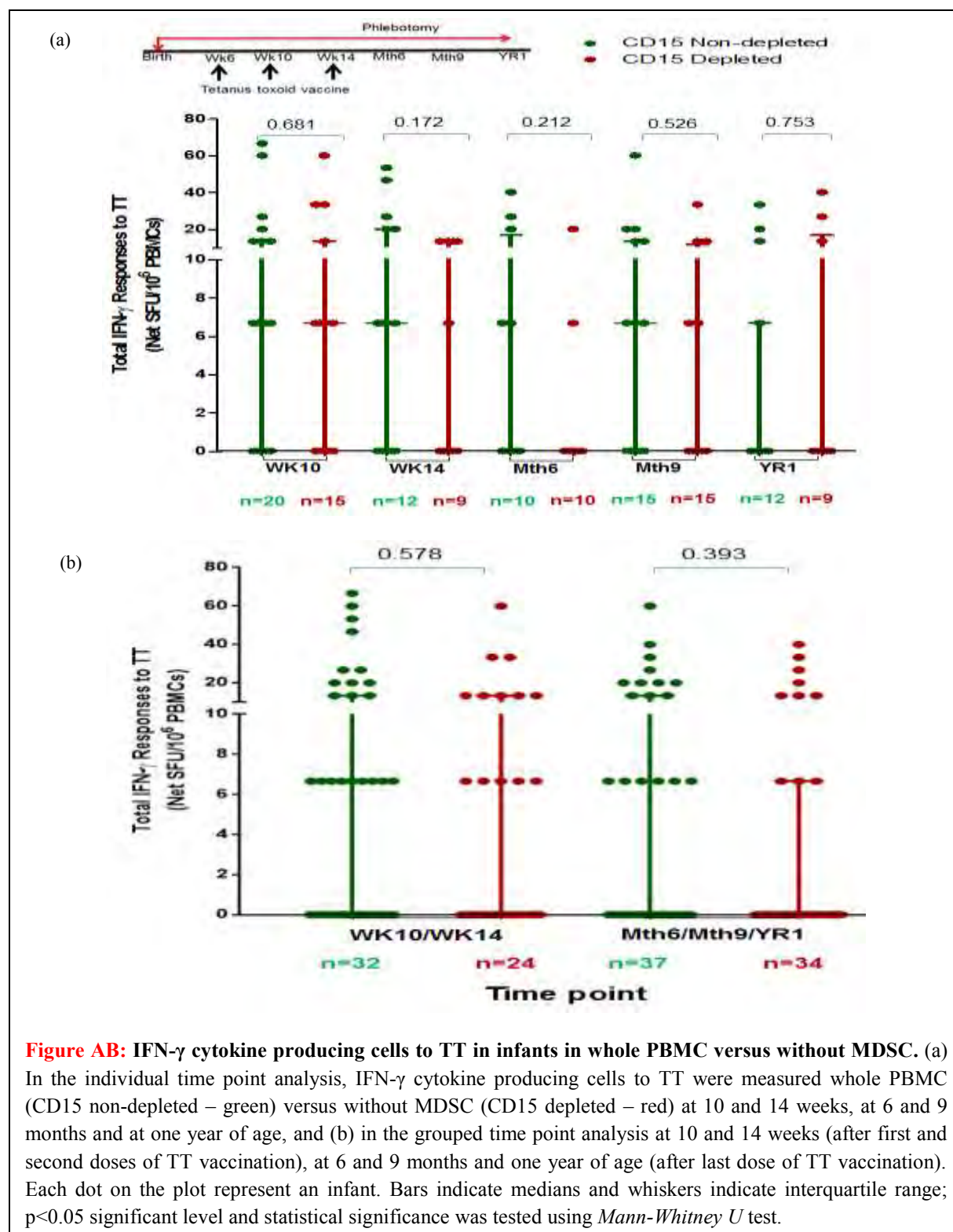
(ii) BCG induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in mothers

In mothers, BCG induced IFN- γ producing cells were evaluated in whole PBMC and without MDSC at delivery and at one year postpartum. As shown in Figure AA, the frequency of cells producing IFN- γ to BCG were not significantly different in mothers after gestation at delivery (median of CD15 non-depleted=20.000 Net SFU/ 10^6 PBMC versus CD15 depleted=13.330 Net SFU/ 10^6 PBMC and $p=0.125$), but were significantly higher before MDSC depletion at one year postpartum (median of CD15 non-depleted=20.000 Net SFU/ 10^6 PBMC versus CD15 depleted=0.000 Net SFU/ 10^6 PBMC and $p=0.037$).



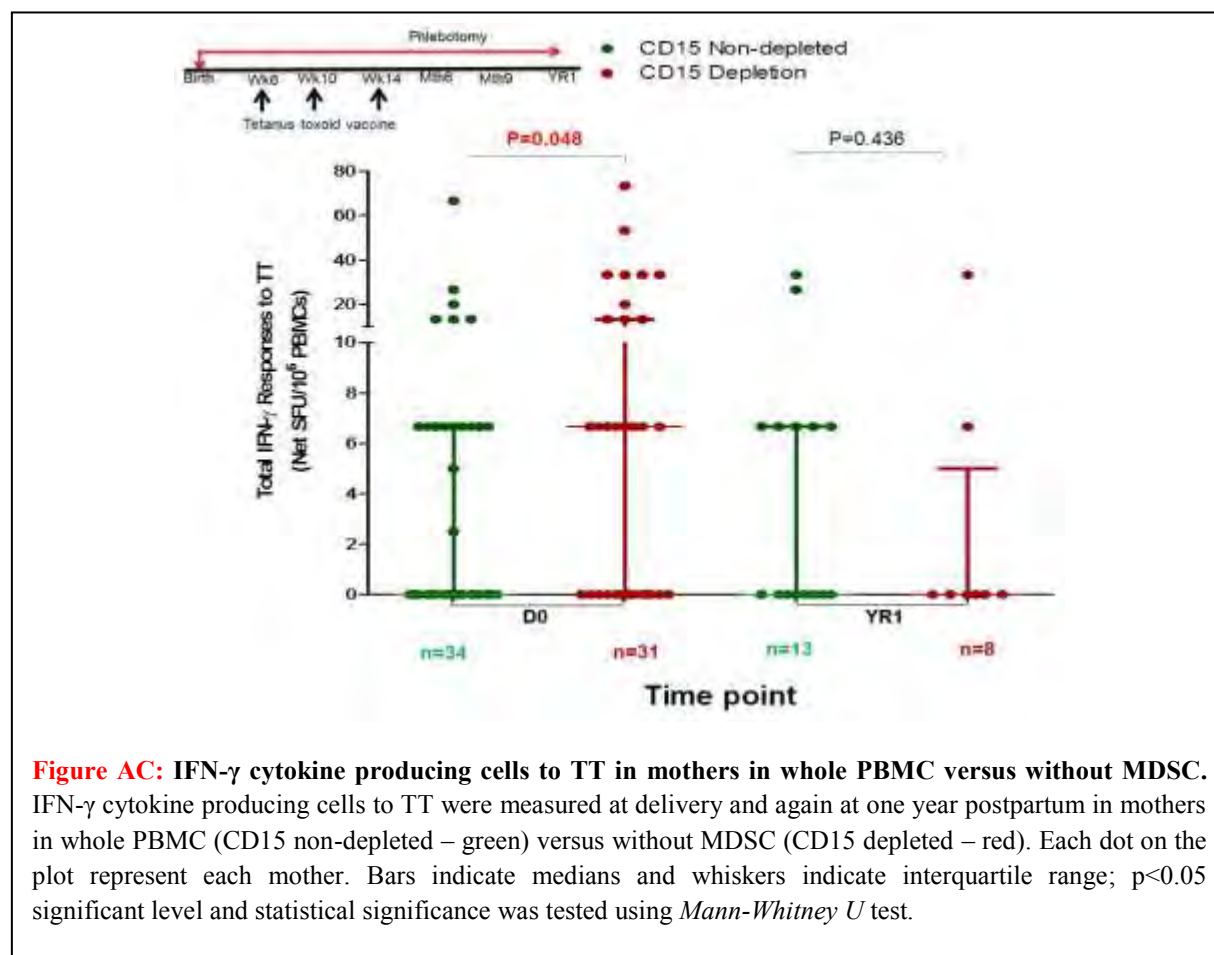
(iii) TT induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in infants

The cells producing IFN- γ to TT were evaluated in whole PBMC and without MDSC in infants at 10 and 14 weeks, at 6 and 9 months, and at one year of age. There was no effect of MDSC depletion at any time point as shown in **Figure AB**. However, IFN- γ cytokine producing cells to TT lasted till one year of age. There was no significant difference (a) at the individual time points between groups at 10 and 14 weeks ($p=0.681$ and $p=0.172$ respectively), at 6 and 9 months ($p=0.212$ and $p=0.526$ respectively) and at one year of age ($p=0.753$); or (b) in the grouped time point analysis at 10 and 14 weeks (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-12.413 versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-11.330; $p=0.578$), and at 6, 9 months and one year (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-11.213, versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-6.141; $p=0.393$). Hence, no major difference in cells producing IFN- γ in whole PBMC and after MDSC depletion at all time points was observed in infants.



(iv) TT induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in mothers

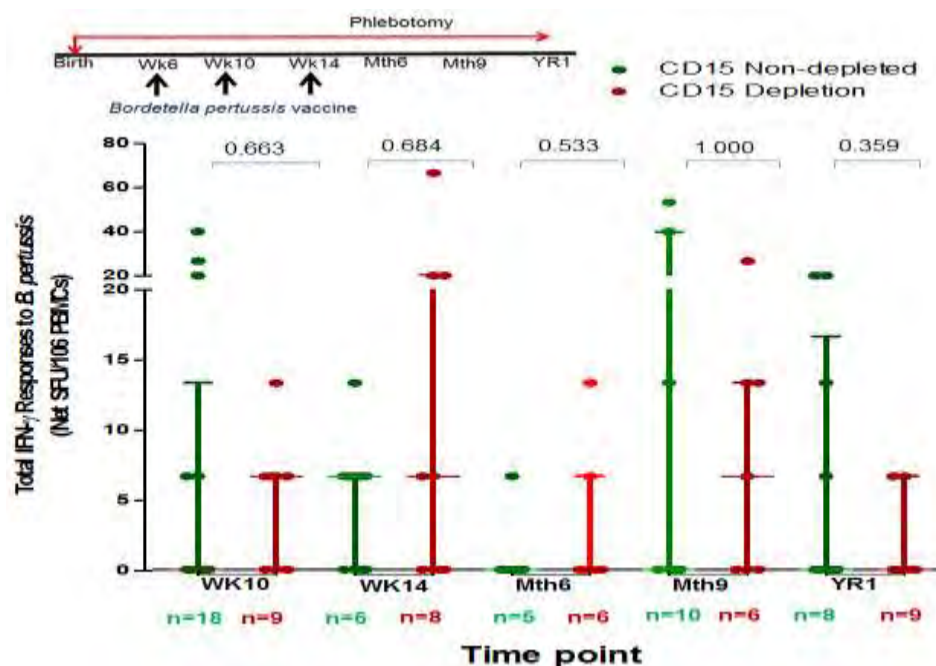
In mothers, TT induced IFN- γ producing cells were evaluated in whole PBMC and without MDSC at delivery and again at one year postpartum. As shown in **Figure AC**, cells producing IFN- γ to TT were significantly lower before MDSC depletion in mothers after gestation at delivery (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC versus CD15 depleted=6.667 Net SFU/ 10^6 PBMC and $p=0.048$), but not at one year postpartum (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-33.330 versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-33.330 and $p=0.436$).



(v) BP induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in infants

The cells producing IFN- γ to BP vaccination were evaluated in whole PBMC and without MDSC in infants at 10 and 14 weeks, at 6 and 9 months, and at one year of age (Figure AD). There was no significant difference (a) at the individual time points between groups at 10 and 14 weeks ($p=0.663$ and $p=0.684$ respectively), at 6 and 9 months ($p=0.533$ and $p=1.000$ respectively) and at one year of age ($p=0.359$); or (b) in the grouped time point analysis after grouping infants at 10 and 14 weeks (median of CD15 non-depleted=3.333 Net SFU/ 10^6 PBMC versus CD15 depleted=6.667 Net SFU/ 10^6 PBMC; $p=0.549$), and at 6, 9 months and one year (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-13.330, versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-6.677; $p=0.907$). Hence, no major difference in cells producing IFN- γ before and after MDSC depletion at all time points was observed in infants.

(a)



(b)

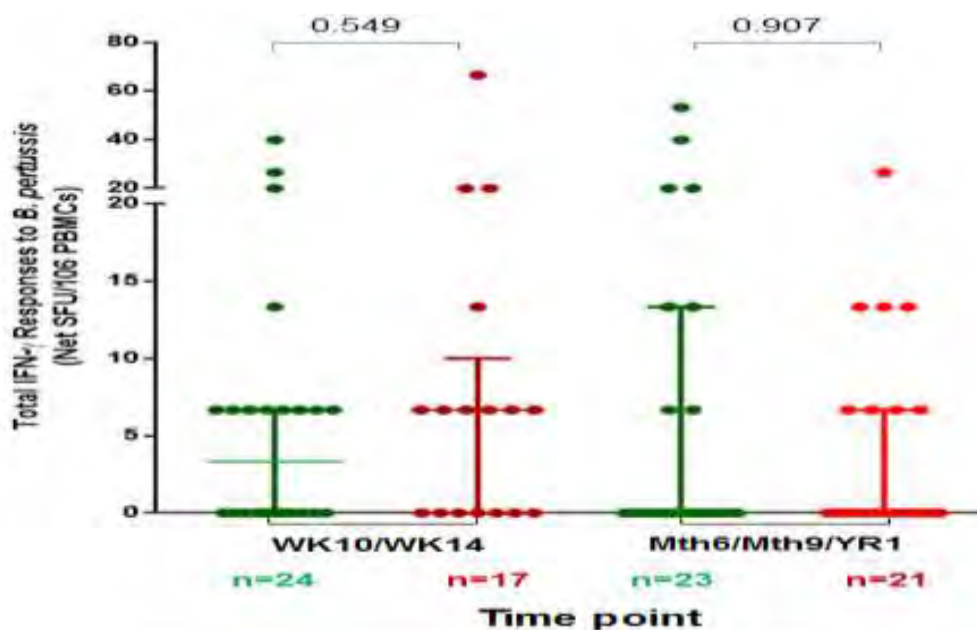
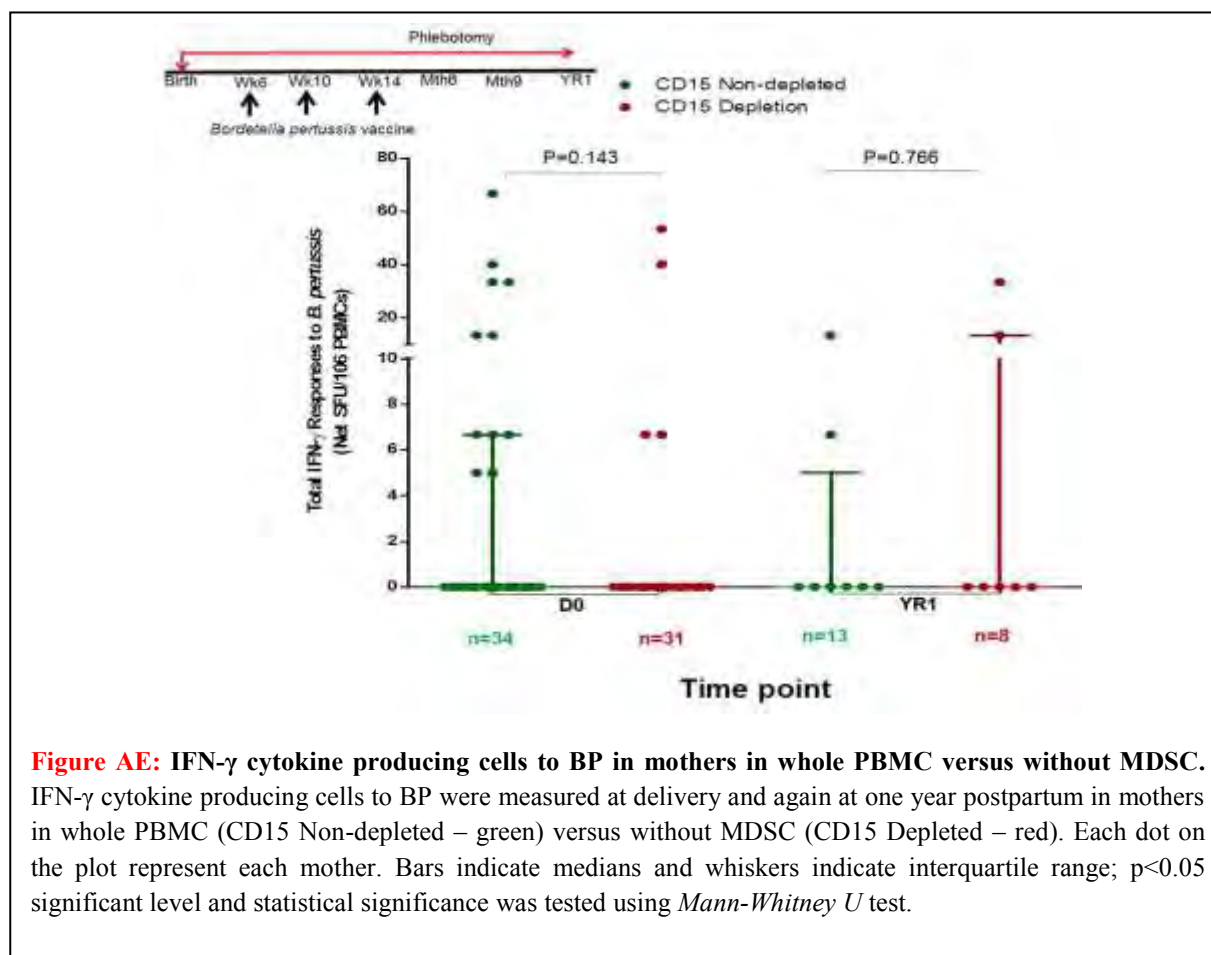


Figure AD: IFN- γ cytokine producing cells to BP in infants in whole PBMC versus without MDSC. (a) In the individual time point analysis, IFN- γ cytokine producing cells to BP were measured in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red) at 10 and 14 weeks, at 6 and 9 months and at one year of age, and (b) in the grouped time point analysis at 10 and 14 weeks (after first and second doses of BP vaccination), at 6 and 9 months and one year of age (after last dose of BP vaccination). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

(vi) BP induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in mothers

In mothers, quantities of BP induced IFN- γ producing cells were evaluated in whole PBMC and without MDSC at delivery and again at one year postpartum. As shown in **Figure AE**, MDSC depletion did not affect IFN- γ production in mothers at either time point (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-66.670 versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-53.330 and $p=0.143$), and at one year postpartum (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-13.330 versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-33.330 and $p=0.766$).



(vii) Hep B induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in infants

The cells producing IFN- γ to Hep B vaccination were evaluated in whole PBMC and without MDSC in infants at 10 and 14 weeks, at 6 and 9 months, and at one year of age. There was no significant difference (a) at the individual time points at 10 and 14 weeks ($p=0.463$ and $p=0.252$ respectively), at 6 and 9 months ($p=0.826$ and $p=0.861$ respectively) and at one year of age ($p=0.361$); or (b) in the grouped time point analysis at 10 and 14 weeks (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-13.333 versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-3.330; $p=0.175$), and at 6, 9 months and one year (median of CD15 non-depleted=0 Net SFU/ 10^6 PBMC, IQR=0-6.333 versus CD15 depleted=0 Net SFU/ 10^6 PBMC, IQR=0-13.330; $p=0.421$) (Figure AF). Hence, no major difference in cells producing IFN- γ to Hep B before and after MDSC depletion at all time points was observed in infants.

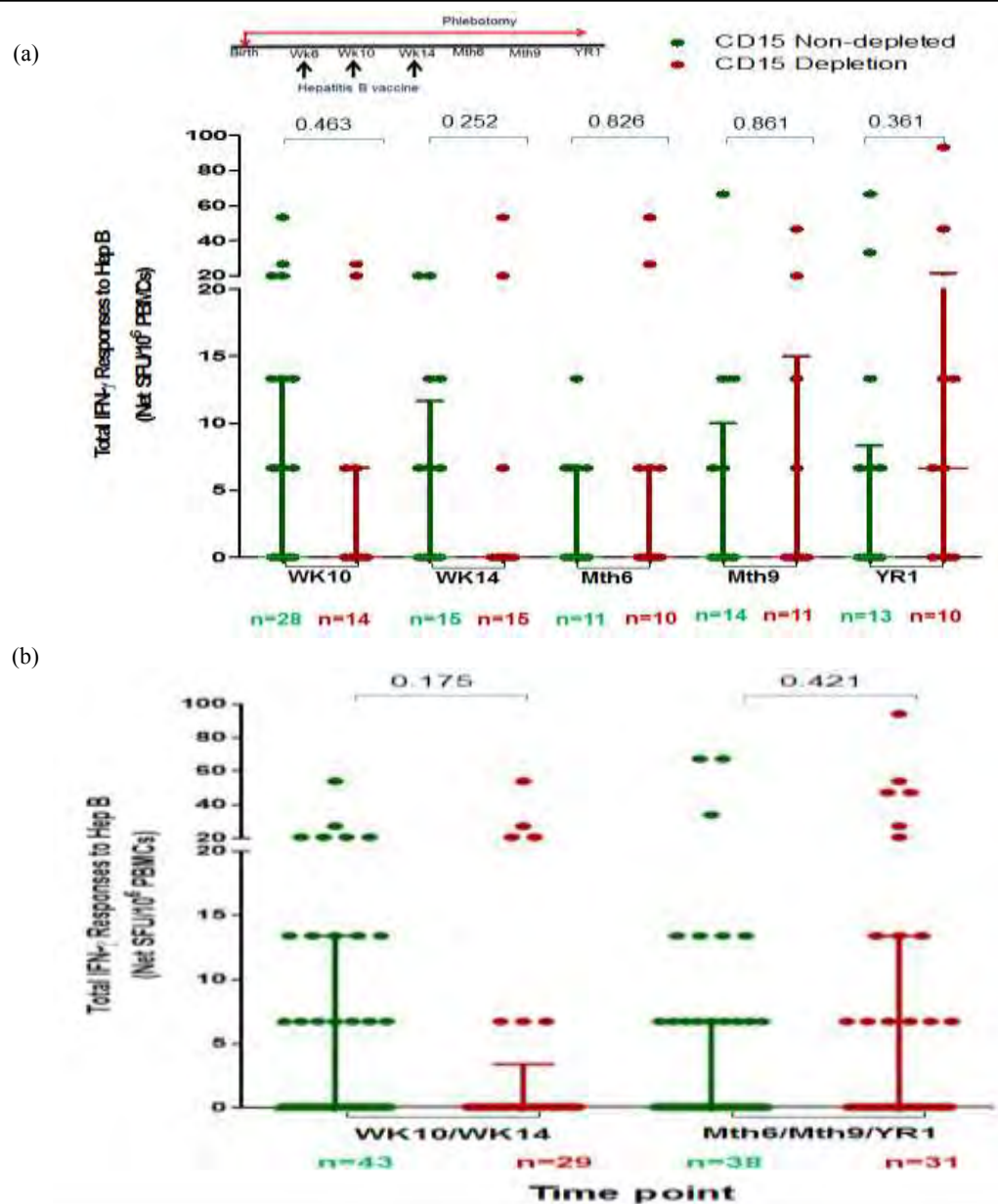
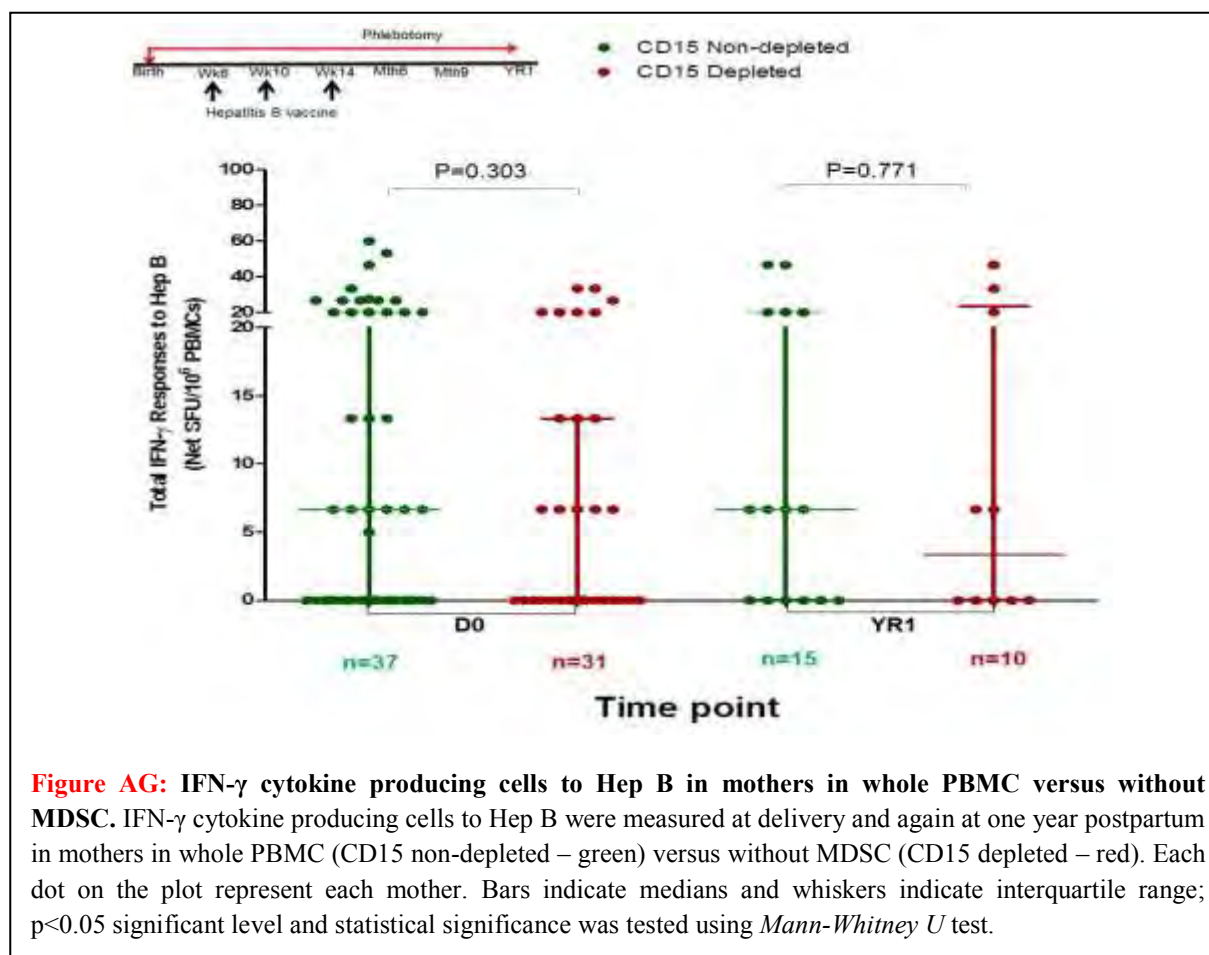


Figure AF: IFN- γ cytokine producing cells to Hep B in infants in whole PBMC versus without MDSC.

(a) In the individual time point analysis, IFN- γ cytokine producing cells to Hep B were measured in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red) at 10 and 14 weeks, at 6 and 9 months and at one year of age, and (b) in the grouped time point analysis at 10 and 14 weeks (after first and second doses of Hep B vaccination), at 6 and 9 months and one year of age (after last dose of Hep B vaccination). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

(viii) Hep B induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in mothers

In mothers, Hep B induced IFN- γ producing cells were evaluated with and without MDSC at delivery and at one year postpartum. As shown in **Figure AG**, cells producing IFN- γ to Hep B were not significantly different with and without MDSC in mothers after gestation at delivery (median of CD15 non-depleted=6.667 Net SFU/10⁶ PBMC versus CD15 depleted=0 Net SFU/10⁶ PBMC and $p=0.303$), and at one year postpartum (median of CD15 non-depleted=6.667 Net SFU/10⁶ PBMC versus CD15 depleted=3.333 Net SFU/10⁶ PBMC and $p=0.771$).



(ix) PHA induced IFN- γ cytokine producing cells in the presence of MDSC versus after MDSC depletion in infants

Phytohaemagglutinin (PHA) has been widely described and its use as a mitogen to trigger T lymphocyte cell division and to activate latent HIV-1 from human peripheral lymphocytes has been well documented (Mire-Sluis *et al.*, 1987) (Gondo-Rey *et al.*, 2001). We employed PHA as a positive control and we also assessed how MDSC would affect cells producing IFN- γ to PHA. We evaluated IFN- γ cytokine producing cells to PHA with and without MDSC in infants at birth, at 6, 10 and 14 weeks, at 6 and 9 months, and at one year of age (Figure AH). IFN- γ cytokine producing cells to PHA lasted till one year and were very strong at all time points. There was no significant difference at birth (median of CD15 non-depleted=353.300 Net SFU/ 10^6 PBMC versus CD15 depleted=390.000 Net SFU/ 10^6 PBMC and $p=0.908$), at 6, 10 and 14 weeks ($p=0.180$, $p=0.561$ and $p=0.333$ respectively), at 6 and 9 months ($p=0.124$ and $p=0.309$ respectively) and at one year of age ($p=0.178$). Hence, no big difference in cells producing IFN- γ before and after MDSC depletion was observed in infants.

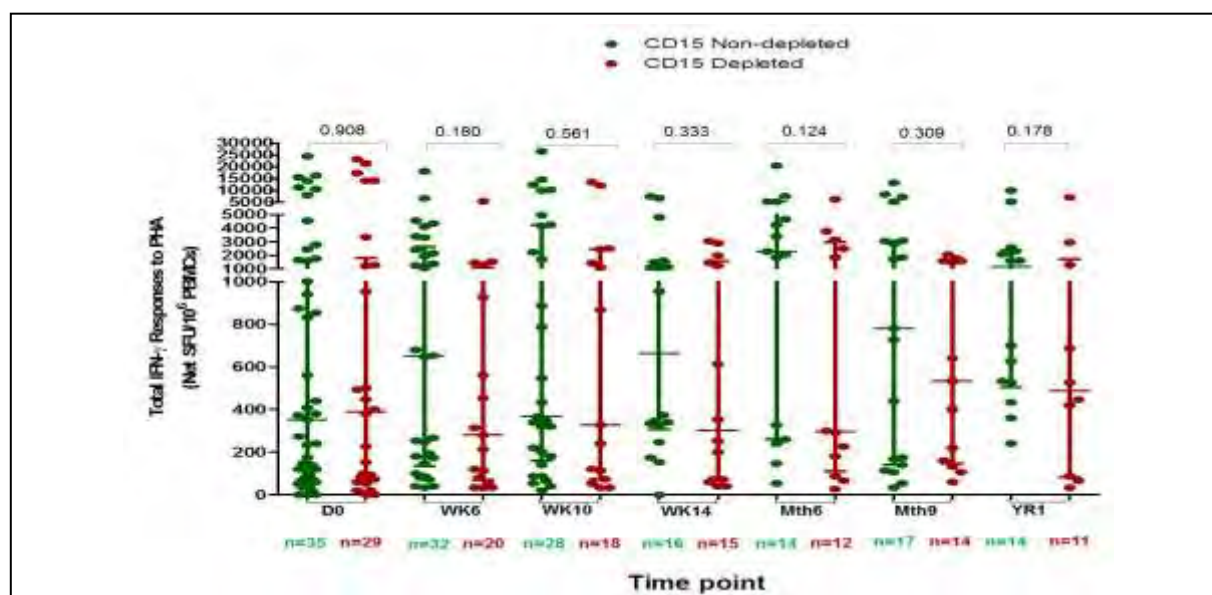


Figure AH: IFN- γ cytokine producing cells to PHA in infants in whole PBMC versus without MDSC. IFN- γ cytokine producing cells to PHA were measured at birth, at 6, 10 and 14 weeks, at 6 and 9 months and at one year postnatal in infants in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red). Each dot on the plot represent an infant. Bars indicate medians and whiskers indicate interquartile range; $p < 0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.

(x) PHA induced IFN- γ cytokine producing cells in whole PBMC versus after MDSC depletion in mothers

In mothers, PHA induced IFN- γ producing cells were compared in whole PBMC and without MDSC at delivery and at one year postpartum. As shown in **Figure AI**, cells producing IFN- γ to PHA were significantly higher in whole PBMC than after MDSC depletion in mothers at delivery (median of CD15 non-depleted=2927.000 Net SFU/ 10^6 PBMC versus CD15 depleted=1947.000 Net SFU/ 10^6 PBMC and $p=0.047$), but not at one year postpartum (median of CD15 non-depleted=1440.000 Net SFU/ 10^6 PBMC versus CD15 depleted=320.000 Net SFU/ 10^6 PBMC and $p=0.061$).

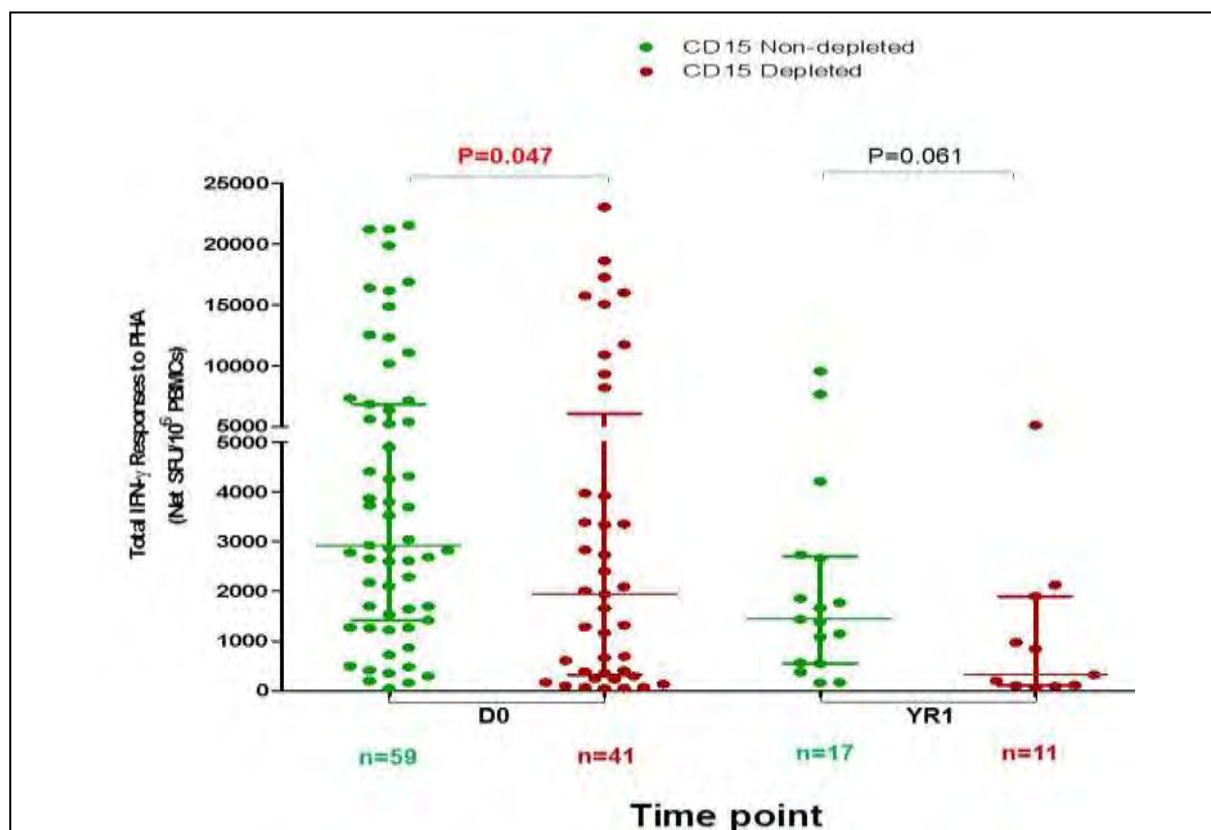


Figure AI: IFN- γ cytokine producing cells to PHA in mothers in whole PBMC versus without MDSC. IFN- γ cytokine producing cells to PHA were measured at delivery and again at one year postpartum in mothers in whole PBMC (CD15 non-depleted – green) versus without MDSC (CD15 depleted – red). Each dot on the plot represent each mother. Bars indicate medians and whiskers indicate interquartile range; $p<0.05$ significant level and statistical significance was tested using *Mann-Whitney U* test.